CONTRIBUTIONS TO THE BIOLOGY OF DIPLOCOCCUS INTRACELLULARIS.

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INTRODUCTION.

Cerebro-spinal meningitis prevailed as an epidemic in Greater New York City during the winter of 1904 and the spring of 1905. The epidemic embraced about 4000 cases of the disease. The deaths numbered 3,429. In the year 1904, 1,403 deaths occurred from meningitis. In the year 1905, the number of cases of the disease reported was 2,755, and the number of deaths, 2,026. The percentage mortality, deduced from the figures for 1905, is 73.5 per cent.

In the spring of 1905, the Health Department of the City of New York appointed a commission to co-operate with the department in investigating the epidemic. As a member of the commission, I undertook an experimental investigation of epidemic meningitis, out of which the following reports have grown. The rather one-sided nature of the studies, which are to be given here, is to be explained, in part, by the allotment of different aspects of the investigation to the several members of the laboratory committee of the commission.[1]

The particular subject of investigation alloted to me was the pathogenic properties of *Diplococcus intracellularis,* and the immunization of animals with a view to obtaining immune sera which might be used for the differentiation of the diplococcus by the agglutination test. In the course of the study of its pathogenic effects, I became impressed with certain biological properties of the *Diplococcus intracellularis* which have not hitherto been recorded.

In a small number of instances only did I isolate the diplococcus

[1] The laboratory committee of the Commission consisted of Drs. W. J. Elser, E. K. Dunham, J. M. Van Cott, Jr., and Simon Flexner.

directly from spinal fluids obtained by lumbar puncture, or from exudates secured at autopsy. I relied for the greater part of the cultures with which I worked upon Dr. E. K. Dunham, Dr. Martha Wollstein, of the Babies Hospital, and Dr. E. P. Bernstein, of Mt. Sinai Hospital. I frequently obtained from Drs. Wollstein and Bernstein cultures in the first and second generation on serum-glucose-agar. Dr. Dunham provided me with many strains of *Diplococcus intracellularis* and a number of diplococci whose properties were not identical with this diplococcus. These latter were obtained, for the most part, from the nose and pharynx. I secured a number of strains of diplococci, which I compared with *Diplococcus intracellularis,* from the nose and throat of healthy persons, and from domestic pets, cats and dogs, owned by persons in whose homes cases of meningitis occurred.[2]

The difficulties of cultivating *Diplococcus intracellularis* were greatly reduced by the employment of sheep serum instead of human serum. For this advantage I am indebted to a suggestion of Dr. W. H. Park, of the Department of Health. Sheep-serum-water, prepared according to Hiss' method, is mixed with a beef infusion agar-agar containing 2 per cent. of glucose. The quantity of sheep-serum water need not exceed $\frac{1}{20}$ to $\frac{1}{10}$ of the volume of the agar. It is added to the sterile and melted agar-agar which is afterwards slanted in test tubes, or allowed to congeal on the expanded surface of 16 ounce Blake bottles. The latter are used for obtaining mass cultures.

The identification of the cultures of *Diplococcus intracellularis,* which I studied, offered no special difficulties. Their origin from

[2] The nasal cavities of the domestic pets were examined by my assistant, Mr. Ward. As a rule, it was necessary to etherize the animals in order to obtain satisfactory "swabs" from the upper nasal mucosa. Several cocci or diplococci decolorizing by Gram's method were obtained but they were readily distinguished from *Diplococcus intracellularis.* Through the kindness of Dr. A. C. Abbott, of Philadelphia, Mr. Ward was enabled to make plate cultures from the upper nasal mucous membrane of fifty inmates of the Philadelphia Hospital. At the time of this investigation meningitis was not epidemic in Philadelphia, and it did not become so afterwards. The inoculated plates were divided with Dr. E. K. Dunham. Neither Dr. Dunham nor Mr. Ward obtained from them any diplococcus which could be confounded with the *intracellularis.* Cocci agreeing with *M. catarrhalis* were, however, obtained from several plates.

the spinal canal of persons suffering from epidemic meningitis was always presumptive evidence of their nature. The diplococci fulfilled the ordinary cultural requirements, and they showed the low degree of viability which has been observed commonly. I quickly learned to lay some stress upon rapidity with which involution occurred in cultures which grew luxuriantly, and, later, I learned to trust the fermentative reactions upon sugars of the diplococcus. Dextrose and maltose are feebly attacked by the diplococcus, and acid is produced. Gas is never liberated, and in a sheep-serum-water-litmus medium, containing the sugars mentioned, reddening, but no coagulation, is produced.[3] Certain other diplococci, not staining by Gram's method, with which *Diplococcus intracellularis* might possible be confused, were either devoid of power to ferment sugars, or they possessed wider or more vigorous fermentative power than the intracellularis.[4]

AUTOLYSIS OF THE DIPLOCOCCUS.

The brief vitality of many cultures of the intracellularis is a point of differential importance. Many strains, grown on a favorable medium, unless transplanted to a fresh medium, do not survive beyond two or three days. Indeed, these transplantations do not succeed, frequently, unless considerable quantities of the growth are carried to the fresh tubes, an indication that only a fraction of the cocci have survived, or are still capable of multiplication. Vigorous fresh cultures of 16 to 24 hours' growth stain sharply and uniformly. On the second day, the cocci show irregularities in staining and size, which irregularities are increased by dessication of the surface of the medium and by other injurious causes. Cultures three days old show marked degenerations, and these increase rapidly with age until, at the end of five or six days, or even earlier, no normal cocci persist. Even with very careful preservation, the great majority of the cocci do not survive beyond this period. A few cocci, in carefully preserved cultures of certain strains, may survive for many

[3] The sugar reactions of the diplococcus have been studied especially by Dr. Dunham and Dr. Elser. (E. K. Dunham, *Jour. Infec. Diseases,* 1906, Supplement 2, p. 10; W. J. Elser, *Jour. Med. Research,* 1906, ix, 89.)

[4] The value and limitation of the agglutination reaction are discussed by Dunham in his report, *Op. cit.*

months. The appearance of old cultures is characteristic. At the beginning of the degeneration, swollen, deeply-stained cocci occur among the smaller but usual forms, which take the stain less intensely than fresh cultures. At this stage, tetradic groups appear, especially among the swollen cocci. As degeneration progresses, loss of staining and disintegration ensue, until, finally, staining power is entirely lost and a formless detritus remains.

The slight viability of *Diplococcus intracellularis* when grown outside the body, and the characteristic changes associated with its dissolution, led me to try the effect of a number of experimental conditions upon these phenomena. The low degree of viability was conceivably due to rapid exhaustion of the medium of growth, at least of that superficial layer of the solid medium upon which growth takes place, or of accumulation of growth products, or of these two factors acting together. The possibility must be kept in mind that the life-history of the diplococcus, within and without the body, may be, at best, a brief one. In this case vigorous-looking cocci would appear in cultures only so long as multiplication was proceeding; and once the cocci have reached the expiration of their brief life-period, disintegrative or involutional changes would set in quickly. Some notion of the influence of exhaustion of the medium and accumulation of metabolic and disintegration products upon multiplication and involution, could be obtained by experiment.

A series of tubes containing the glucose-sheep-serum agar was inoculated with the diplococcus by smearing the slanted surfaces with a recent culture. After twenty-four hours' growth at 37° C., the surfaces were washed with successive quantities of sterile salt solution. Some of the tubes were now returned to the thermostat, and others were returned after the surfaces had been covered with a layer of sheep-serum water and then drained free of the excess. Previous tests had shown me that the diplococcus does not grow in sheep-serum water to which glucose has not been added. Examination of the cultures twenty-four to forty-eight hours later showed that growth had taken place in all the tubes, was less abundant than in the first instance, and presented a filmy, granular appearance. Microscopically, typical diplococci were present; at the expiration of forty-eight hours in the thermostat, the number of involution-forms was large.

Owing to the ease and rapidity with which the diplococcus can be made to undergo dissolution (see below), its soluble disintegration products are readily obtained. These products were tested for their effect upon the growth and vitality of the diplococci.

The large flat surface of medium in a one-pint Blake bottle was inoculated with a culture (654) of *Diplococcus intracellularis*. An abundant growth was obtained in twenty-four hours. About 15 c.c. of sterile 0.85 per cent. salt-solution were poured over the surface, and a few drops of toluol added. The bottle was returned to the thermostat until the next day, at which time an almost clear fluid, devoid of toluol, remained overlying the agar surface from which the growth had disappeared. Cover-glass preparations showed disintegration of all the cocci. The fluid was centrifugalized until clear, and distributed to melted glucose agar as follows: each tube contained 8 c.c. of the melted agar (cooled to 60° C.) to which were added 0.5 c.c., 0.8 c.c., 1.0 c.c., 1.5 c.c. and 2.0 c.c., respectively, of the "extract" of the cocci. The tubes were allowed to cool in the slanting position and each was inoculated with a vigorous culture (656) of the diplococcus. The tabulation shows the result. First observation after twenty-four hours.

Quantity of Extract.	Visible Growth.	Microscopical Examination.	Subsequent History.
0.5 c.c.	Moderate.	Pale staining, relatively large, Gram neg. diplococci.	Spread over surface.
0.8 c.c.	None.		Re-inoculated with fresh culture.
1.0 c.c.	Moderate.	Pale staining, relatively large, Gram neg. diplococci.	Spread over surface.
1.5 c.c.	None.		Re-inoculated.
2.0 c.c.	None.		Re-inoculated.

The second observation was made at the expiration of a second twenty-four hours. The spread colonies now yielded an abundant growth; the re-inoculated tubes all gave growths in separate colonies which, on microscopical examination, showed vigorous cocci except in case of the tube receiving 2 c.c. of the "extract" in which the dozen colonies of diplococci presented a swollen and involutional appearance. At the lapse of another twenty-four hours, all the cultures had begun to degenerate or showed more advanced degeneration, the latter being most marked in the tube with 2 c.c. of the "extract." Transplantations to fresh serum-agar surfaces gave growths in all instances.

From these observations, it would appear as if the brief vitality of the cultures grown outside the body is not to be ascribed directly to the exhaustion of the medium of growth, or the accumulation of injurious products of growth, in the short period of twenty-four to forty-eight hours. It may be safely stated that the quantity of disintegration products added to the tubes in the form of "extract" greatly exceeded the possible accumulation under ordinary conditions of cultivation. The higher concentrations of the "extract" undoubtedly inhibited the growth, and exerted an harmful influ-

ence on the morphological appearances of the diplococci. But as the cultures could still be transplanted successfully at the expiration of forty-eight to seventy-two hours, at which period the experiment was terminated, they may be taken to have retained their vitality quite unimpaired..

It has been observed repeatedly that cultures of *Diplococcus intracellularis* tend to survive longer when kept constantly at the thermostat temperature than when kept at varying room temperatures, or in the ice box. That cultures tend to die quickly when placed in the ice box was noted accidentally by me early in this investigation. A series of tests was carried on in order to determine whether any differences could be noted depending, first, upon the concentration of the suspension of cocci kept on ice, and next, upon intermittent variations in temperature between 37° C. and 2° C., as compared with a constant thermostat and refrigerator temperature.

Two heavy suspensions in salt solution of diplococci (596 and 598) were kept in the refrigerator, at a temperature of about 2° C., for five days, at the expiration of which time vigorous and abundant growths were still obtained on transplantation. This result has an interesting bearing on the influence of concentration of the suspension of the cocci on their disintegration at higher temperatures.

Three tubes containing vigorous twenty-hour-old cultures of diplococcus (656) were treated as follows: (*a*) was kept constantly at 37° C., (*b*) was alternated daily between the thermostat and refrigerator (2° C.), and (*c*) was kept constantly in the refrigerator. Experiment began November 8. First transplantation November 10. (*a*) and (*b*) abundant growths; (*c*) moderate growth. Second transplantation November 12. (*a*) and (*b*) good growths; (*c*) small number of separate colonies. Third transplantation November 13. (*a*) and (*b*) good growths; (*c*) no growth.

From these tests certain conclusions can be drawn. In the course of growth of the diplococcus, the medium (if solid) is not exhausted of all nutritive value for the coccus in twenty-four to forty-eight hours; and the products of disintegration of the diplococcus are inhibitive to further multiplication of the coccus only when they have accumulated in large quantities. It would indeed appear as if such an accumulation of growth products as to prevent further development could probably not occur in the course of ordinary vegetative existence of the organism; and it is safe to infer that the short vitality of the coccus in cultures is not due directly to the injurious action of these substances. The tests fur-

ther bring out the fact that a fresh and vigorous culture upon a slanting solid surface succumbs quickly when kept constantly at a temperature just above zero, and is less injured by the wide variation of temperatures between 2° C. and 37° C. It could, therefore, be shown that cold is inherently injurious to the cocci, and the injury is inflicted not at once that the low temperature is reached, or equally upon the cocci exposed to it. This fact has an important bearing upon the enzymotic activities of the diplococcus to be described presently. In view of the ease and certainty with which solid cultures are destroyed by cold, the greater resistance displayed by highly concentrated suspensions of the cocci in salt solution is noteworthy. Salt solution, even when changed with washings from the surface of a solid medium, probably does not permit any multiplication of the diplococci; besides which any increase is excluded, in this experiment, by the low temperature. That so many of the cocci survived in the strong suspensions may be due merely to greater vitality of a small number of the cocci, or it may, on the other hand, be due directly to the concentration of the fluid which conceivably exercises a protective influence. This factor of concentration of suspension of cocci is very important in respect to the preservation of the cocci under the conditions of higher temperature when enzymotic action may be presumed to come actively into play, and hence it was desirable to determine whether it played a really important part at the lower temperatures.

A milky suspension of a culture "Smith," twenty hours old, on sheep-serum agar was made in salt solution. The suspension was divided in three parts and used for an experiment at 37° C., 2° C. and —5° C.

The first experiment to be related was made at 2° C. Three tubes were charged with the suspension: (1) was the original strength; (2) contained 0.5 c.c. of the original suspension and 1.0 c.c. salt solution, and (3) contained 0.1 c.c. of the original suspension and 1.0 c.c. salt solution. The first observation was made at the end of twenty-four hours. Cover-glass preparations showed the cocci to be well-preserved in (1), somewhat less sharply-staining in (2) and (3). At the expiration of the second twenty-four hour period, at which time cultures were made, cover-glass preparations showed in all the tubes a moderate reduction in sharpness of outlines and staining of the cocci. The results of the cultivation are important. (1) showed a solid growth of diplococci covering the midportion of the tube; (2) gave sixteen characteristic colonies; (3) gave no growth whatever. Approximately the same volume of suspension, but not the same number of diplococci, was transplanted in each tube.

The deduction from the previous test and this experiment is obvious: cold is injurious to the cocci and destroys cocci in a weak more quickly than in a more highly concentrated suspension. It will not be profitable to discuss at this juncture whether the survival in the concentrated suspension is due directly to its numerical superiority in cocci or indirectly to the existence of more resistant cocci which, as the whole number is more numerous, are also more numerously represented. In even the weakest suspension (3), the cocci transplanted must have been innumerable.

Still further depression of the temperature appears to exert even a greater injury to the cocci. Suspensions of the cocci which were placed, at the same time, at — 5° C. failed entirely to grow when transplanted, forty-eight hours later, to a favorable medium, although the morphological changes in the cocci were relatively slight.

It was stated that salt solution is not a medium of growth and multiplication of the cocci. A series of tests was made with salt solution and other media in order to determine the fate of the diplococci introduced into them. The details of the experiment need not, perhaps, be given. A strain of the diplococcus, which by reason of long cultivation outside the body was able to grow feebly on plain agar, was employed. Salt solution (0.85 per cent.), distilled water, and sheep-serum water were inoculated with small quantities of the culture and examined twenty hours later. The salt solution and distilled water showed diplococci in a good state of preservation; the sheep-serum water showed no diplococci. At the expiration of the next twenty-four hours, those in the distilled water were in part fragmented and decolorized, those in the other media remaining unchanged. Growths could not be recovered from any of the fluids.

The observation of the persistence of the diplococci in a dead state in salt solution and water for about forty-eight hours without suffering dissolution is interesting when compared with what takes place, under similar conditions, when the suspensions are heavier. I had observed, in the course of experiments on the virulence of the diplococcus that a concentrated salt suspension tended to go off in virulence for guinea-pigs in a few hours, and, as compared with

the state of preservation of solid cultures of the same age, they were more advanced in involution. This observation led me to a study of the influence of concentrations of salt suspension upon the vitality and destruction of the diplococcus, which gave some interesting results.

If a fresh culture, on a favorable medium, is suspended in salt solution to a milky mixture, and the latter is kept at 37° C., the greater number of the cocci introduced will be so degenerated at the end of twelve to fourteen hours as to be almost unrecognizable. As the suspension is diluted progressively, the degree of degeneration and disintegration diminishes. The relative number of diplococci which survive in these suspensions can be determined by cultures at any period. A single experiment will be given here.

A milky suspension of culture (610) was made in salt solution. Microscopical examination showed innumerable diplococci of characteristic appearance. A series of test tubes were charged as follows and placed in 37° C.

(1) Original suspension.	Micro. exam. after 24 hours; disintegration, shadows, fragments.	After 48 hours: no coccal forms preserved.
(2) Original suspension 1.0 c.c.; salt sol. 1.0 c.c.	Micro. exam. after 24 hours: disintegration slightly less than (1).	After 48 hours: some shadowy coccal forms.
(3) Original suspension 0.5 c.c.: salt sol. 1.0 c.c.	Micro. exam. after 24 hours: disintegration less than (2).	After 48 hours: shadows and deep staining cocci remain.
(4) Original suspension 0.1 c.c.; salt sol. 1.0 c.c.	Micro. exam. after 24 hours: many shadows, no fragmentation.	After 48 hours: best preservation of series.

Cultures made on sheep-serum agar from the supensions kept at 37° C. for twenty hours gave: Original suspension = no growth; dilution (2) = twelve separate diplococcus colonies; dilution (3) = large number of separate and confluent colonies; dilution (4) = two hundred separate colonies.

If this experiment, which was carried out at the thermostat temperature, be compared with the one similar to it in all respects, except that the suspensions were kept two degrees above the freezing point, it will be seen at once that, however the salt solutions may affect the viability of the diplococcus, the influence of concentration on the viability, is diametrically different under the two sets of conditions given. In other words, at the lower temperature more diplococci survive in the concentrated than in the weaker suspen-

sions, while at the higher temperature, more of them survive in the weaker than in the higher concentration. A glance at the microscopical preparation gives a clew to this difference of behavior: while at the refrigerator temperature the diplococci are not markedly disintegrated, at the thermostat temperature degree of disintegration and concentration go hand in hand. Hence, while the diplococci may lose capacity to grow without losing, at the same time, their form and staining reactions, it is probable that once these latter are lost, viability is also lost. Cold, like salt solution, is inherently hurtful to *Diplococcus intracellularis;* but, besides the injury which the salt solution alone inflicts, another detrimental factor comes into action at the thermostat temperature.

Before considering this factor, the question of the manner in which the salt solution acts may be taken up. It must be borne in mind that as compared with the salt strength of the medium on which the diplococcus is cultivated, the 0.85 per cent. salt solution is hypertonic. A. Fischer[5] has shown that passage from weak into more concentrated salt solution produced in bacteria sometimes plasmolysis, sometimes plasmoptysis, and sometimes neither of these changes. Does, therefore, the change of medium in this case bring out plasmoptytic alterations of the diplococci? Three facts speak against the changes described as being plasmoptytic. The first is the influence of concentration of the bacterial emulsion, the second is the effect of low as compared with higher temperatures, and the third, now to be described, is the influence of a salt solution of 0.9 per cent. concentration containing calcium and potassium chloride. If suspensions of the diplococcus are made in Ringer's solution the viability of the coccus is greatly prolonged as compared with the survival in the salt solution, or, what is even more remarkable, upon the sheep-serum agar. A series of Ringer's solution's suspensions of the diplococcus of three concentrations: (1) Milky suspension, (2) first suspension 0.5 c.c. and Ringer's solution 1.0 c.c., (3) first suspension 0.1 c.c. and Ringer's solution 1.0 c.c., kept at 37° C. survive many days. I have observed that on the twelfth day a considerable number of the diplococci in the original milky suspension was still viable, a smaller number was still viable

[5] *Zeitschr. f. Hygiene u. Infectionskrank.*, 1900, xxxv, 1.

in the suspension of medium concentration, while no growth was obtained after the fifth day from the lowest concentration. All the diplococci had died out in the original suspension on the fifteenth day. This experiment establishes several important points: it proves that the death of the diplococcus is not caused by hypertonicity of medium alone, by starvation alone, by degree of concentration alone, or by a naturally brief period of vitality. If frequent microscopical examinations of the suspensions in Ringer's fluid are made a gradual dissolution of the diplococci can be made out, but the disintegration progresses slowly, as compared with the effect of salt solution, and the sharp distinction between the different concentrations is lost. I have noticed in those suspensions which gave subcultures a change in appearance of a number of the diplococci which may be connected with the power of survival. These individuals, which are somewhat larger than the average, and take on a deep safranin stain, displaying a high degree of refraction, exhibit a greater resistance to the disintegrative influences. I had already observed these morphological forms in my experiments upon the destructive action of exudates upon the diplococcus, and the question occurred to me whether they represent a more enduring type of the micro-organism. I am unable, at present, to answer this question.

A simple experiment has shown that the addition of a salt of calcium to an otherwise suitable culture medium will greatly increase the period of viability of the diplococcus which has grown upon it. If calcium carbonate is added to the sheep-serum water agar, a given culture will survive many weeks. A rough experiment showed that at the end of five weeks a strain which otherwise survives, on the medium minus the calcium salt, only two, three or four days, was still alive and able to yield abundant sub-cultures. While at the end of five or six days, the original cultures show no preserved, but only disintegrated diplococci; the growth on this new medium two weeks old still showed innumerable diplococci of faint staining power.

The experiment at 37° C. with the Ringer's solution indicated that the survival of diplococci was not necessarily in inverse ratio to the concentration of the suspension, but was in ratio to the con-

centration. In this respect the influence of Ringer's fluid is widely different from that of salt solution, and agrees with the effect of cold on the survival of the diplococcus in salt suspensions. It was deemed desirable to ascertain whether cold, as such, exercised as injurious an influence upon the diplococcus suspended in Ringer's solution as it was shown to exercise on agar cultures and on salt suspensions. Diplococci suspended in Ringer's fluid and kept at 2° C. survived eight to ten days, which is about three times as long as they survived under the other conditions. This period is behind that of survival at 37° C., whence it follows that cold as such is more injurious than warmth. Diplococci could be subcultured longer from the stronger than from the weaker suspensions kept at 2° C.; and as the power to grow in subcultures was lost disintegration of the diplococci was observed to take place extensively. Exceptions to this rule may be observed. A highly concentrated suspension in Ringer's fluid of a vigorous looking diplococcus culture failed, in one instance, to survive 24 hours at 2° C.

It must, therefore, be concluded that salt solution is directly injurious to *Diplococcus intracellularis,* as it has been shown by Jacques Loeb[6] and others to be poisonous to many animal cells; and it must be belived that the toxic effects of sodium chloride can be neutralized by certain calcium and potassium salts. Although the salt solution causes directly the death of the diplococci, it would appear that it is not to it, but to some other agency, to which must be ascribed the disintegration which rapidly overtaxes the dead micro-organisms.

The nature of this agency is rendered fairly evident from the microscopical appearances presented by the suspensions: it would seem to be enzymotic. The manner of dissolution of the diplococci suggests this, and the enzyme is doubtless contained in the bacterial cells. As enzymes act with energy not upon highly vitalized, but upon dead or partly devitalized cells, the question arises whether in the case of the diplococcus the enzyme can act immediately as a devitalizing as it can as a dissolving agency. It has been shown that in weak salt suspensions the diplococci survive and retain their morphological integrity longer than in stronger suspensions; and

[6] *Biochemische Zeitschrift,* 1906, ii, 81.

hence, it may be conceived that while the charges of enzyme which individual diplococci carry may be inadequate both to destroy and to disintegrate them, that in the more concentrated suspensions the enzyme liberated from a larger number of disintegrating micro-organisms, may exert a devitalizing effect. The intracellular enzyme is, therefore, a dissolvent of dead diplococci, and in certain states of concentration a poison for living ones.

The action of the enzyme upon the bacterial cells can be accelerated by the employment of chemical and physical agents which serve to kill the diplococci without, at the same time, seriously injuring the enzyme. Heat, carefully applied, and toluol have been used to accomplish this purpose. Suspensions of the diplococcus heated for thirty minutes to the temperature of 60° C. are killed and still capable of undergoing disintegration or autolysis. The ultimate result is the same whether the suspension has been made in salt or Ringer's solution; while in point of rapidity the dissolution is somewhat slower than in strong unheated suspensions in salt solution. Heating to 65° C. and higher temperatures, reduces the capacity of the diplococcus to undergo rapid disintegration, probably because the enzyme is weakened or destroyed.

If to a strong salt suspension of the diplococcus toluol be added and the mixture kept at 37° C., the morphological changes indicative of autolysis begin to be evident in one hour, to be pronounced in two hours, and nearly complete in four hours. Ringer's fluid has no marked influence on this form of autolysis. It is interesting to note that the degree of concentration of the suspension influences the result in the presence as in the absence of toluol. Even the most concentrated salt suspensions alone do not show appreciable changes in the morphology of the diplococcus at the expiration of four hours at 37° C. The tabulation which follows brings out the main facts.

If Series I and II in the succeeding tabulation are closely scrutinized, such differences in degree of disintegration can be made out as to indicate that toluol not only accelerates autolysis of the cocci, but it tends also to make it more complete and to minimize, but not to set aside entirely, the effect of the degree of concentration upon the final disintegration.

A milky suspension of diplococcus (656), showing under the mircroscope innumerable diplococci, was prepared. Series I consisted of salt suspensions of cocci alone, Series II of salt suspensions to which toluol was added. The examination was made after eighteen hours at 37° C.

SERIES I.

Original suspension.	Extensive disintegration; small number of coccal forms still visible.
Original suspension, 1.0 c.c.; salt sol. 1.0 c.c.	Less disintegration than previous one.
Original suspension, 0.5 c.c.; salt sol. 1.0 c.c.	Less disintegration than previous one.
Original suspension, 0.2 c.c.; salt sol. 1.0 c.c.	Fair degree of preservation of cocci.
Original suspension, 0.1 c.c.; salt sol. 1.0 c.c.	About same degree of preservation as previous one.

SERIES II.

Original suspension + toluol.	Complete disintegration.
Original suspension 1.0 c.c.; salt sol. 1.0 c.c. + toluol.	Complete disintegration.
Original suspension 0.5 c.c.; salt sol. 1.0 c.c. + toluol.	Disintegration incomplete; pale and irregular coccal forms visible.
Original suspension 0.2 c.c.; salt sol. 1.0 c.c. + toluol.	Somewhat better preservation than previous one.
Original suspension 0.1 c.c.; salt sol. 1.0 c.c. + toluol.	Better preservation than previous one.

Jacques Loeb[7] has drawn attention on several occasions to the restraining influence of potassium cyanide upon intracellular enzymotic activity. The diplococcus offered, therefore, the opportunity to study the effects of this chemical body upon an active autolytic ferment under the conditions of purity of culture of microorganic cells—conditions probably not obtainable with the cells of higher living forms.

The experiments were made with different strengths of the cyanide and suspensions of the diplococci. Potassium cyanide in 1/200 per cent. solution kills the diplococci outright. If now the diplococci, in watery suspensions, are left in contact with the cyanide, either with or without the addition of toluol, the organisms do not disintegrate, and suffer only reduction in their affinity for

[7] *Biochemische Zeitschrift*, 1906, i, 183.

dyes. That enzyme activity is not wholly suppressed by the cyanide would seem to be indicated by the better state of preservation of the cocci in the dilute suspensions. The tabulation (Series III) brings out this difference. The suspensions and examination of the diplococcus were made at the same time as those of Series I and II of the preceding tabulations.

SERIES III.

KCN 1/20% 1.0 c.c., susp. cocci 1.0 c.c.	Outlines of cocci pale but visible; possibly reduction in numbers.
KCN 1/20% 1.0 c.c., susp. cocci 0.5 c.c.	Fair preservation of the cocci; no reduction in numbers.
KCN 1/20% 1.0 c.c., susp. cocci 0.2 c.c.	Good preservation of the cocci.
KCN 1/20% 1.0 c.c., susp. cocci 0.1 c.c.	Very good preservation; outline of cocci not perfectly distinct.
KCN 1/20% 1.0 c.c., susp. cocci 0.01 c.c.	The same as previous one.

This experiment shows that potassium cyanide possesses the power of preventing or diminishing autolysis of the dead diplococci. In the case of the higher concentrations it is even possible that a greater quantity of the cyanide may have wholly suppressed autolysis. Doubtless many other chemical agents possess this power of suppressing autolysis of the diplococcus; and hence it was necessary to ascertain whether the removal of the cyanide will admit the enzyme to reassert its enzymotic action, as, according to Loeb, happens in the case of the eggs of the sea-urchin. If, therefore, the cyanide acts by holding the enzyme in check and not by destroying it, or changing the cell-substance, so as to make it permanently resistant to the action of the enzyme, the diplococci should be restored approximately to their normal condition of autolysis upon the removal of the cyanide.

Ten cubic centimeters of a strong suspension of the diplococcus in salt solution were mixed with one cubic centimeter of a one twentieth per cent. potassium cyanide solution, and the mixture kept at 37° C. for one hour. The cocci and fluid were separated by centrifugalization and the coccal residue washed twice with 10 c.c. of salt solution. The sediment now showed a large number of well-preserved, deeply-staining diplococci. The sediment was resuspended in salt solution and divided into two portions, to one of which toluol was added. They were placed at 37° C. and examined at the end of twenty-four hours and forty-eight hours. No marked difference was noticed in the toluol and non-

toluolized specimen. The diplococci appeared reduced in number, evident disintegration was in progress, and the sharpness of staining had been lost at the first examination. At the second examination, the staining was still more feeble, but no other change was noticeable.

From the experiments, it can be stated that the diplococci, after treatment with the cyanide can be partially restored to their previous state of autolysis. The experiment is not conclusive of the actual degree of restoration possible, since the disintegration of the cocci was incomplete in the experiment. The concentration of the resuspension, which was not high, may explain the imperfect disintegration. It would, indeed, be possible to conduct this experiment quantitatively and ascertain the degree of permanent injury exercised by the cyanide upon the diplococcus; but the object of the experiment in establishing the restraining action of potassium cyanide upon the autolytic enzyme, and the possibility of setting this action aside by mechanical removal of the poison, was attained by the imperfect experiment.

The existence of an autolytic enzyme in *Diplococcus intracellularis* capable of destroying its own cell-substance having now been established, the next step was to ascertain whether the enzymotic action was specific and limited to the diplococcus, or whether it could be exerted upon other bacterial cells. It is known that the diplococcus does not produce a proteolytic enzyme acting upon and dissolving gelatine and coagulated serum. Hence, its enzyme differs materially from the enzymes secreted by certain liquefying bacteria. It seems quite certain that the rapid and striking morphological changes taking place in cultures of the diplococcus are caused, directly or indirectly, by the intracellular enzyme; and it may, therefore, in view of this fact and the related ones already described, be presumed that the diplococcal enzyme is able to break down the complex structures contained in living micro-organisms.

The enzymotic action of the diplococcus was tested upon the two classes of bacteria represented by those staining and those decolorizing by Gram's method. Among the latter, *Bacillus typhosus, Bacillus coli communis, Bacillus pyocyaneus, Micrococcus catarrhalis* and two unidentified Gram negative cocci from the monkey's nose were tested; while among the former *Staphylococcus aureus* and *Bacillus anthracis* were chosen.

The experiments to determine this point were made in the following manner: Fresh growths upon agar-agar surfaces were suspended in salt solution to a milky emulsion which was divided into two equal parts. To one part an equal volume of salt solution, and to the other the same volume of a milky suspension of *Diplococcus intracellularis* was added. Control cover-glass preparations were made immediately and double-stained with gentian-violet and safranin (Gram's method).

Toluol was now added, and the tubes placed at 37° C. under rubber caps. The examinations were made by means of cover glass preparations (Gram's method), usually at twenty-four hour intervals. A few detailed results follow.

Non-Gram coccus from monkey. (*a*) Controls: Original suspension shows many sharply-staining cocci, single and in pairs; mixed suspensions of coccus and diplococcus permit easy distinction by the morphological differences. After twenty-four hours: Original suspension shows no solution or disintegration of the cocci; outlines of the cocci not quite sharp. Mixed suspension: All the diplococci have disintegrated; the larger cocci from the monkey have lost stainable substance and have run together into small masses in which the individual outlines are indistinct. After forty-eight hours: Original suspension, cocci somewhat paler and cohering slightly. Mixed suspension: Reduction in number of cocci; those remaining appear as shadows. The non-Gram coccus (*b*) gave results which were almost identical with those of (*a*).

B. typhosus and *B. coli communis* in milky suspensions under toluol, are somewhat altered in appearance at the end of forty-eight hours at 37° C.; while in the presence of an emulsion of the diplococcus, such as was used with the non-Gram cocci from the monkey, they are completely or almost completely destroyed in this period. At the expiration of the first twenty-four hours, the disintegration is advanced.

Bacillus pyocyaneus.—The control under toluol showed, at the end of twenty-four hours, no reduction in number, but less deep staining than the preparation made immediately after suspension. The suspension with the diplococcus showed, at this time, reduced numbers, and only shadowy remains of bacilli. At the expiration of the second twenty-four hour period, the control tube still showed many stainable bacilli (number apparently less than in original suspension), while the mixed tube containing the two organisms was completely disintegrated.

Two cultures of *Micrococcus catarrhalis,* which were found to be quite resistant under toluol, were found to disintegrate quickly (in twenty-four hours) under the influence of the suspension of the diplococcus.

Staphylococcus aureus.—Control (under toluol) showed no appreciable change after several days at 37° C. The mixed suspension of staphylococcus and diplococcus was examined at twenty-four hour periods for five days. After twenty-four hours, there is reduction in numbers of the staphylococci, although many

still remain. In size these are larger than in the control tube, and the violet staining is less intense. In the succeeding days, the number of cocci further diminished, the cocci coalesced into clumps, and showed still further reduction in power to hold the violet stain. Here and there throughout the preparation, transitions from staphylococci holding the violet stain imperfectly to staphylococci which had taken up the counter-stain (safranin) were observed. At the expiration of the experiment, not all the staphylococci had disappeared or had been decolorized.

Anthrax bacillus twenty hours old on slant agar; no spores visible. Suspended as before: First observation after twenty-four hours. Control (under toluol): Partial and moderate degree of plasmolysis. The threads and felted masses of bacilli show clearly an outer-stained (safranin) cell-wall and an inner-stained (violet) cell-substance. The inner substance is somewhat fragmented and irregularly contoured. Mixed suspension: Advanced plasmolysis and loss, in high degree, of Gram-staining inner substance. The outer cell-membrane is completely gone in many places, and the fragmented or partially dissolved inner substance lies free. Second observation, after forty-eight hours: Control unchanged. Mixed suspension: Practically complete decolorization of the threads, with very few fragmented remains of violet-staining inner cell-substance.[2]

It is established by the foregoing series of tests that the enzymotic action of the diplococcus can be exercised on a considerable variety of bacteria, and the observations are not without interest as indicating that micro-organisms, such as *Staphylococcus aureus, Bacillus anthracis,* and *Bacillus pyocyaneus,* in spite of their energetic proteolytic functions, show less power to cause disintegration of their own protoplasm than is effected by *Diplococcus intracellularis.* This difference points to a distinction between autolytic and proteolytic bacterial enzymes. The above tests also showed that a great difference exists in regard to the ease and completeness with which this hetero-disintegration of bacterial cells is produced by the diplococcus. Of the bacteria studied which were at all subject to the enzyme *Staphylococcus aureus* offered the greatest resistance; and I think it not improbable that the Gram-positive bacteria in general may prove to be less readily broken down by the diplococcus enzyme than the Gram negative bacteria.[3] Eventually, the bacteria which produce proteolytic enzymes, and, indeed other bacteria, suffer more or less complete disintegration. In the case of *Bacillus*

[2] The destruction of *Staphylococcus aureus* was incomplete, the change in *Bacillus anthracis* was one of degree of disintegration; the changes in the other bacteria were more striking. No effect was noticed in tests with *Bacillus tuberculosis* and *Bacillus Moelleri* (timothy bacillus).

pyocyaneus, in salt suspension in toluol, the time required for disintegration is, at most, a few days. But the slower forms of involution and degeneration observed in artificial cultures, may or may not be of the nature of the autolytic changes described. These involutional alterations are probably of extremely subtile nature; but even here enzyme activities of quite low grade may be the chief agencies at work.

The heat lability of the enzyme has been studied in two ways. First, the temperature at which the diplococcus is prevented from undergoing characteristic autolysis was determined. Next, the

The suspended contents in sheep-serum water of surface growths from two-pint Blake bottles were distributed among ten test tubes; each tube received about 6 c.c. The tubes were, with the exception of the controls, heated for thirty minutes to temperatures ranging from 55° C. to 90° C. Cultures were made to determine whether any diplococci remained viable. Only the control (unheated) tubes gave growth. Cover-glass preparations were made from the control tubes, and from the heated tubes immediately after heating and at the subsequent periods. The tabulation gives the results:

Suspension.	Immed. Microscop. Appearance.	Microscop. Appearance 24 Hours Later.	Sedimentation After 3 Days.
Control.	Well-preserved cocci.	Marked degeneration; diminution of cocci.	Cloudy; no sediment.
Toluol control	Well-preserved cocci.	A few minute, pale fragments remain.	Almost clear; no sediment.
55°C.	Cocci stain less sharply.	Some cocci preserved; many shadows and fragments.	Almost clear; mod. sediment.
60°C.	Cocci stain less sharply.	Some cocci preserved; many shadows and fragments.	Almost clear; mod. sediment.
65°C.	Some frag. and decol. cocci.	Somewhat more fragmentation than at 55°C.	Almost transparent; much sediment.
70°C.	Some frag. and decol. cocci.	Forms better preserved than at 65°C.	Almost transparent; much sediment.
75°C.	Some decolorization.	Preservation very good.	Almost transparent; much sediment.
80°C.	Considerable fragmentation.	Preservation good, but much irregularity in staining.	Almost transparent; much sediment.
85°C.	Frag. and apparent diminution in number.	Staining deep; no shadows; irregular forms.	Almost transparent; much sediment.
90°C.	Frag., diminution in number; decolorization.	Forms pale; no further diminution.	Almost transparent; much sediment.

temperature required to render the autolysate of the diplococcus inactive upon other bacterial species was ascertained.

A temperature of 55° C., maintained for thirty minutes, kills the diplococcus, but it does not suffice to remove its capacity for self-digestion. Even after exposure, for the same period, to a temperature of 60° C., the diplococcus undergoes autolysis. This test was several times repeated with different strains of the diplococcus and autolysis was always observed to occur, although somewhat less quickly and perfectly than in unheated coccal suspensions under tuluol. Toluol accelerates, I believe, dissolution, by causing plasmolysis and thus promoting the disintegrative effect of the enzyme. The thermal death points of cocci and enzyme are widely separated. Temperatures of 40° C. to 45° C. are capable, if maintained some hours, of killing the diplococci, while the enzyme does not suffer injury under the same conditions. Temperatures of 65° C. and upwards, while causing more or less immediate plasmolysis (or plasmoptysis) prevent further disintegration due to a vigorous action of the enzyme. As the tabulation indicates, two criteria can be used to determine the point of heat-destruction of the enzyme: first, the degree of dissolution shown by the microscope, and next the amount of sediment appearing in the tubes. The two effects stand in a quantitative inverse ratio to each other. The greater the amount of sediment, the smaller the amount of disintegration of the diplococcus as shown by the microscope. Hence the gross test is well adapted to the study of the lability of the enzyme, since it permits of the elimination of any error arising from mere physical or mechanical change in the diplococci.

To ascertain whether the temperature at which self-digestion of the diplococcus is inhibited represents the temperature of heat-destruction of the enzyme, another series of tests was made.

The point of heat injury or destruction of the enzymotic fluid was determined by preparing, in pint Blake bottles, mass cultures of the diplococcus and causing them to autolyze at 37° C. for forty-eight hours in salt solution and in water under toluol. At the expiration of this period the toluol had evaporated. The salt autolysate was distributed in test tubes, of which some were heated to 70°, 80°, 90° and 100° C. for thirty minutes, and some remained unheated. A coagulum formed in the tubes heated to 90° and 100° C. The watery autolysate was not heated. The contents of all the tubes were centrifugalized; and except

those heated to 90° and 100° C., which became transparent, the fluids remained opalescent. Twenty hours' growths of *B. coli communis, B. typhosus* and *B. anthracis* were suspended in Ringer's fluid to milky emulsions. The tests were made as follows: A set of test tubes was charged with the unheated salt and water autolysates and the salt autolysate heated as described. To the control tube an equal amount of salt solution was added. An approximately equal amount of the milky bacterial emulsion was now placed into each tube, toluol was added, and the mixtures bept at 37° C. for forty-eight hours. Two examinations at tweny-four-hour periods were made. The result in the case of *B. coli communis* was definite: the control salt suspension showed the bacilli swollen, pale and shadowy, but not disintegrated; the salt and water unheated autolysates showed complete disintegration of the bacilli; the autolysate heated to 70° C. was scarcely to be distinguished in effect from the unheated; while the autolysates heated to 80° C. and higher produced about the same degree of change as the salt control. The result in the case of *Bacillus typhosus* was disappointing, as, in this experiment, the salt control as well as the autolysate mixtures disintegrated completely. A perceptible difference was manifest in the degree in which *Bacillus anthracis* disintegrates in toluolized salt solution and in a mixture of equal parts of salt and Ringer's solution. The addition of the latter reduced the spontaneous disintegration. In like manner the unheated autolysates are less active destructive agents where Ringer's fluid is present, as compared with their action in plain salt solution. And yet the disintegration was greater when the unheated autolysate was present. The autolysate heated to 70° C. and 80° C. produced less change, and that heated to 90° C. and 100° C. still less, than took place in the unheated salt and Ringer's fluid control.

While this experiment is not perfectly sharply cut, it yet indicates that heating the fluid autolysate to 70° C. and above reduces its powers of causing disintegration of certain bacteria.

PATHOGENICITY OF THE DIPLOCOCCUS.

Diplococcus intracellurais is, as tested on laboratory animals, a micro-organism of low and variable pathogenic action. I have studied its action upon mice, guinea-pigs, rabbits and monkeys. Different strains of the diplococcus were injected into different regions of the body to determine the fatal doses and the pathological effects produced by the micro-organism. In the case of the monkey the inoculations were made directly into the spinal canal, in order to reproduce the symptoms and lesions of cerebro-spinal meningitis in man. The experiments to be related immediately refer to the action of the diplococcus upon the animals mentioned, except the monkeys. The experiments on monkeys form the subject of a separate communication.

A considerable number of strains of *Diplococcus intracellularis* have been tested for virulence upon mice and guinea-pigs. In all the later experiments upon the virulence of the micro-organisms I preferred to employ small guinea-pigs, as being more susceptible to the action of the diplococcus. The difference in resistance to infection in guinea-pigs, depending upon weight, is considerable. Guinea-pigs of 175 to 200 grams weight have proved highly susceptible to the diplococcus, compared to the resistance displayed by pigs weighing 350 to 400 grams. Mice of about 15 grams weight often withstand larger doses of the diplococcus than the smaller pigs.

Not all cultures of the diplococcus, even in the first generations, are even moderately pathogenic for guinea-pigs and mice, although it is always possible to kill these animals by inoculating an excessively large quantity of cultures of low virulence. Speaking generally, the freshly isolated cultures are much more virulent than cultures grown for a period on artificial media. Occasionally, a strain of the diplococcus will retain its virulence for many months, but, as a rule, this power becomes greatly diminished, or is lost, after the first transplantations extending over a few weeks. I have worked with many cultures which deteriorated in a few days, and one culture (Smith) that retained its virulence for many months, to lose it in the end, and another (656) that displayed no diminution in activity after several months cultivation on sheep-serum agar. The "Smith" culture was passed through many mice, guinea-pigs and monkeys during its active period; but once its power was lost, it could not be restored to virulence by passage through mice or guinea-pigs. It still yields, however, by autolysis, an active extract and can be used as an adjuvant to increase activity of other living cultures.

In any comparison of virulence little weight can, I think, be laid upon the absolute quantities of diplococci injected. The animals are, on the whole, so refractory that the number of diplococci that must be injected to produce striking results is, even with the most active cultures, very large. Very little accuracy could in my work be secured through the use of a standard *oese,* for the reason that the contained water in the surface growth was subject to wide

fluctuations, depending upon the strain of diplococcus, composition of the culture-medium, and other external factors.[9] Hence, I substituted for the *oese* a suspension of the diplococci, incubated for a uniform period (usually 20 hours) upon a standard medium (7 c.c. glucose-agar to 1 c.c. sheep-serum water). The suspensions exhibited a degree of opacity which was approximately equal. Select-

No. of Pig.	Weight in Grams.	Denomination and Quantity of Culture.	Result.
113	165	0.1 c.c. No. 654.	Survived.
119	160	0.2 c.c. "	"
121	177	0.5 c.c. "	Died 9 P.M. same evening.
122	192	0.1 c.c. No. 88.	Survived.
127	175	0.2 c.c. "	Died.
128	182	0.5 c.c. "	"
145	197	0.1 c.c. Buck.	Survived.
150	195	0.2 c.c. "	"
159	170	0.5 c.c. "	Died.
161	165	0.1 c.c. No. 9.	Survived.
162	175	0.2 c.c. "	"
163	166	0.5 c.c. "	"
164	161	0.1 c.c. Connolly.	Survived.
166	190	0.2 c.c. "	"
172	195	0.5 c.c. "	Died.

[9] An attempt was made with four cultures of the diplococcus to increase the virulence by growing the diplococci in a glucose-bouillon medium containing one twentieth of its volume of immune goat serum. For the controls normal goat serum was employed. Each culture was passed at twenty-four-hour intervals through ten successive tubes of medium. The tenth cultures were injected into the peritoneal cavity of mice. As the tabulation indicates, no augmentation was accomplished. The immune serum caused agglutination of the growing diplococci. The mice dying in twenty-four hours showed, as is usual, general infection with the diplococcus. Mr. Herbert Ward assisted me in these experiments.

Quantity of Culture Injected.		Immune Series Result.	Normal Series Result.
Kepp culture.	3 c.c.	Died in 15 hours.	Died in 10 days.
	2 c.c.	Survived.	Survived.
	1 c.c.	"	Died in 10 days.
	0.5 c.c.	"	Survived.
	0.1 c.c.	Died in 5 days.	Died in 10 days.
Behren culture.	3 c.c.	Died in 15 hours.	Died in 24 hours.
	2 c.c.	Survived.	Died in 20 hours.
	1 c.c.	Died in 24 hours.	Survived.
	0.5 c.c.	Survived.	"

ing a lot of guinea-pigs of about equal weight approximately uniform quantities of the cultures could be injected.

The preceding tabulation will suffice to show the method of testing comparative virulence and give the results obtained in a typical experiment.

One agar-slant culture of each strain was suspended in about 3 c.c. of salt solution. Injections made intraperitoneally at 5 P. M. The pigs succumbed during the night or the next morning.

As salt solution is evidently an active poison for the diplococcus, and as sheep-serum water favors, when added to an otherwise suitable medium, the growth and preservation of the diplococcus, an experiment was made to ascertain whether suspending the diplococci in one or the other fluid influenced the pathogenic result. The experiment was made with a culture on sheep-serum agar eighteen hours old. The suspensions were prepared at 11 A. M., and the injections given intraperitoneally at 4 P. M. In point of concentration the salt suspension was, as indicated by the microscopical field, considerably the heavier.

No. of Pig.	Weight in Grams.	Quantity of Suspension Injected.	Result.
102	230	1.0 c.c. salt.	Died on second day.
121	267	0.5 c.c. "	Survived.
134	260	1.0 c.c. serum.	Died on first day.
130	270	0.5 c.c. "	Died on second day.
190	115	0.2 c.c. "	Died on first day.

This result merely suggests that as compared with a sheep-serum water suspension the salt suspension of diplococci suffers a more rapid deterioration. In certain experiments on immunity which I carried out I observed that it was unsafe to carry the salt suspensions of the diplococci over, from one day to the next, without making a new determination of the strength. The apparently better preservative action of serum-water over salt solution is the more remarkable in that the serum water is not a favorable medium of growth for the diplococcus. Indeed, many strains are unable to develop in it alone; and the transplanted diplococci, which do not multiply, soon disappear. If equally heavy suspensions, say an agar-slant surface growth per 4 c.c. of fluid, of the diplococcus are made in salt solution and sheep-serum water and kept at 37° C.,

the disintegration is more advanced at the end of twenty-four hours in the salt than in the serum-water.[10]

In studying the pathogenic action of the diplococcus salt suspensions were invariably used. Where a large number of animals was to be inoculated, the diplococcus was grown on the flat agar-covered surface of a one-pint Blake bottle. A suspension of the growth in 12 c.c. of fluid was made of which the fatal dose varied, according to the virulence of the diplococcus, from 0.1 to 0.5 cubic centimeter for small guinea-pigs, by intra-peritoneal inoculation. Death from these doses occurred, as a rule, in less than twenty-four hours. Active cultures, in this strength of suspension, may in the smallest dose given cause death in from eight to ten hours. A culture of which a dose of 0.5 c.c. requires twenty-four hours to cause death is one of low virulence. In the course of any large series of experiments, irregularities in the reaction of guinea-pigs to inoculation occur. On the whole, the irregularities occasioned by greater or less resistance on the part of the small pigs are remarkably few. It has, indeed, rarely happened that a guinea-pig of a series died in four hours and another survived thirty-six hours. But these irregularities can, when required, as in tests of immunity, be ruled out by making duplicate inoculations. Care should be exercised not to employ guinea-pigs weighing less than 150 to 160 grams, the most useful weights being about 200 grams. Among the pigs surviving inoculation immediately, it happened, now and then, that death took place after several days, or a few weeks, and was preceded by a state of great emaciation. No constant pathological condition, the emaciation excepted, was found in these animals. The peritoneum was usually clear, and no diplococci remained there or elsewhere in the body.

One of the earliest visible results of the inoculations is a marked reduction in temperature. This is associated, at times, with the characteristic external appearance of sick guinea-pigs: the tendency to avoid the light, and to sit crouched in a corner with the hair erected. The abdominal muscles become tense and hard and the abdomen distended. The irritative effects of the inoculation upon

[10] The directly toxic action of sodium chloride upon the diplococcus has already been described.

the intestine are associated occasionally with prolapsus of the rectum which condition is followed invariably by death. The rapid onset and high degree of reduction of temperature following inoculation permit frequently the prediction in two or three hours of the probable outcome. Hence, it is possible, in many cases, to determine in a short time the result of tests upon virulence of the diplococci, the protective value of immune sera, etc. In testing immune sera it is necessary in each instance to make a preliminary estimation of the activity of the organisms, since the concentration of the suspension and the degree of virulence are such variable factors; and as the suspensions cannot be kept in salt solution for twenty-four hours without changing, and should be injected as soon as possible after preparation, it is an advantage to have this relatively safe guide to further inoculations of the temperature depression. The tabulation will exhibit the effects described.

No. of Pig.	Weight in Grams.	Amounts of Inoc. 12 M.	Temp. Before Inoc.	Temp. and Condition 3 P. M.	Result.
113	170	0.1 c.c. suspension.	38° C.	35.3°C. abdom. tense.	Died 12 M. next day.
119	160	0.2 c.c. suspension.	37.6° C.	34.1°C. " "	Found dead 7 A.M. next day.
121	186	0.3 c.c. suspension.	38.1° C.	35.1°C. " "	Died 9:30 P.M. same day.
122	170	0.5 c.c. suspension.	38.1° C.	34.8°C. " "	Died 9:30 P.M. same day.

As a control for this series the following tabulation will suffice. The temperature of the pigs prior to inoculation was about 38° C. The quantities of emulsion of the several strains of diplococci injected ranged from 0.1 to 0.5 cubic centimeters. The injections

Weight in Grams.	Quantity of Suspension Injected.	Temp. after Inoc.	Result.
165	0.1 c.c.	36° C.	Survived.
160	0.2 c.c.	37.2° C.	"
182	0.5 c.c.	35.6° C.	Died.
165	0.1 c.c.	35.4° C.	Survived.
175	0.2 c.c.	38.6° C.	"
166	0.5 c.c.	38.4° C.	"

were made at 6 P. M., and the temperature of the surviving pigs taken the next morning.

The value of the tabulation immediately preceding consists, first,

in showing that the temperature depression is less in cases in which the inoculation is not fatal, and second, the degree of resistance of the individual pigs is a factor to be reckoned with. The bracketed series illustrates the latter point: the same suspension of diplococcus was injected into the three pigs, and the pig which received the smallest dose showed the greatest depression of temperature. A similar effect has been noted in other cases, but, on the whole, is rather exceptional.

The fate of the diplococci injected into the peritoneal cavity in guinea-pigs depends upon several conditions: the quantity of diplococci injected, the virulence of the diplococci, somewhat upon the individual resistance of the pig, since this is a factor in prolonging or diminishing the period of survival, and the extraneous condition of elapsed time between death and autopsy. As will appear later, the diplococci are not highly resistant to the destructive action of the fluids of the body whether they act upon them in the peritoneal cavity or in the test-tube. Some experiments tend to show that the more virulent diplococci resist the disintegrative action of the fluids in the body longer than the less virulent ones. Comparison of the conditions arising in the peritoneum, depending upon the quantities and the virulence of the diplococci injected, offers a possible explanation of this difference.

The more virulent the diplococcus the shorter is the period of survival after inoculation. Guinea-pigs which succumb in less than eighteen hours—and many succumb to injection of virulent diplococci in ten to fourteen hours—tend to develop a peritoneal exudate poor in leucocytes; while the pigs which survive longer, *i. e.,* from twenty-four to thirty-six hours, tend to exhibit turbid exudates rich in leucocytes. No absolute ratio between the number of leucocytes in the exudate and the degree of disintegration of the diplococci exists; but a relative ratio seems to exist. With an increasing period of survival there is greater emigration of leucocytes and also greater disintegration of diplococci. Hence, the degree of emigration of leucocytes comes to be a measure, in part, of the degree of virulence of the diplococci. The relation can, however, be disturbed either by the inoculation of very large quantities of little virulent, or sublethal doses of the more virulent diplococci, as

might indeed be expected. It is, however, to be pointed out that large overdoses of the weaker diplococci can be readily disintegrated in the peritoneal cavity, even though emigration of leucocytes is largely prevented by the excessive dose. From this fact the result may be deduced that the destruction of the diplococci does not take place necessarily within leucocytes, but can be accomplished by fluid inflammatory exudates alone. As will be seen presently, test-tube experiments bear out this deduction. There is similarity in the pathological conditions met with in cerebro-spinal meningitis in man and in the experimental infection in guinea-pigs. In both species the immediate local reaction to the presence of the diplococcus in the serous cavities is an exudation of fluid, the character and quantity of which varies greatly, perhaps with the quality of the infecting diplococcus. The less cellular fluids often contain the greater number of diplococci of well-preserved appearance which are readily cultivated; while the more cellular fluids often show fewer diplococci and yield cultures with greater difficulty. No striking relation need exist between the virulence of the diplococcus as displayed for man and animals, since the inherent qualities in the germ which determine pathogenicity are often quite dissimilar for different classes of animals.

I have pointed out that some relation between virulence of diplococcus, period of survival of the inoculated guinea-pigs and the emigration of leucocytes exists, and it remains to add that phagocytosis, in the peritoneal cavity, while a variable factor is a fairly constant one. When emigration of leucocytes is at all marked, even with an almost entire preservation of the diplococci, phagocytosis is occurring. When there has been considerable, although not total, disappearance of the free diplococci, phagocytosis is likely to be more abundant. It rarely happens that all the diplococci are within phagocytes unless a small number only of those injected remain. On the other hand, when overdoses of little virulent diplococci are injected they may disappear as stated almost wholly from the peritoneal cavity although the emigration of leucocytes may have been entirely suppressed; or, as is also possible, the emigrated leucocytes may have undergone complete lysis. Hence, I have concluded first, that phagocytosis, in this experimental disease, is a

function of emigration but not a measure of it, and second, the disappearance of the diplococci from the peritoneal cavity does not depend wholly on phagocytosis. I believe, indeed, that the diplococci can be removed, without direct intervention of phagocytes, through self-digestion and digestive action of the inflammatory exudate. The ease with which the diplococcus undergoes autolysis has already been dealt with; and that the inflammatory exudate exerts actively destructive effects upon the diplococcus will be shown presently.

Leucocytes are numerous in every fibrinous exudate, and frequently, in the omentum, when they may be sparse in the peritoneal fluid. Speaking generally, the clear peritoneal fluids contain few leucocytes relatively, and the turbid fluids contain many. The leucocytes of the omentum are large and pale, and those of the peritoneal fluid small and dense. Their nuclei are often irregular in form, or fragmented. Diplococci are common in the protoplasm and rare in the nuclei, and they show all transitions from well-preserved, sharply-staining organisms to fragments of cocci and swollen, metachromatic bodies. Degenerations are exceedingly frequent both in the leucocytes and in the diplococci. Many leucocytes, even those devoid of diplococci, show nuclear fragmentation and vacuolization, while the markedly degenerated diplococci are commonly intracellular. The number of diplococci in the leucocytes is as variable in the guinea-pig as it is in man. Occasionally one finds that the injected diplococci have undergone agglutination in the peritoneal fluid. Where the injected diplococci were quite uniformly distributed in the salt suspension, cover slip preparations from the inflammatory exudate show the micro-organisms to be in smaller and larger clumps, in which condition they may be taken up by phagocytes.

Since in cerebro-spinal meningitis in man cultivation experiments may fail to yield the diplococcus although the microscope shows it to be present, attention may be called to similar failures with guinea-pigs. In the case of these animals the period of survival after inoculation affects the result, since, if the autopsies are made soon after death, those pigs succumbing earliest give the greater number of successful cultivations. But if the autopsies are delayed and par-

tial decomposition of the body has already set in, or if the dead pigs have been kept for many hours in the refrigerator, the chances of successful cultivation of the diplococcus are greatly reduced. I have noticed that the diplococci taken from pigs dead some hours, which have not grown on planting on a good medium, show irregularities in staining indicative of beginning dissolution: while a pig which had died at about the same time from a corresponding injection of the diplococcus, but which had been kept in the refrigerator during the same number of hours (six to eight), showed the diplococci in a better state of preservation, although they too may not have grown in the tubes. In the first instance, the diplococci have succumbed, probably, to the combined action of inflammatory exudate and the products of decomposition; and in the second, they were probably killed by the low temperature (vide supra). Should the former pigs remain somewhat longer at the fairly warm room temperature, the diplococci may disappear entirely post mortem. Hence, failure to grow upon artificial culture media may depend upon changes suffered by the diplococci in the infected body, or in the body after death. I may anticipate, in this place, an observation made upon the medulla oblongata of a monkey which succumbed to an intra-spinal injection of the diplococcus. The medulla was covered with a recent purulent exudate from which diplococci were easily cultivated. Two portions were excised of which one was placed at 37° C. and the other at 2° C. over night. Neither of these now gave growths, and the cover slip preparations which originally showed numerous diplococci now showed many fewer in the exudate from the section of tissue placed in the thermostat; no such diminution occurred in the cooled tissue.

In view of the manner in which *Diplococcus intracellularis* acts in causing death of guinea-pigs, it may be assumed that its effects are produced by a poison liberated from the bacterial bodies, probably through disintegration, and not to a secreted extracellular poison. Multiplication on the part of the diplococci is not at all essential to the production of the peculiar symptoms and lesions. Even though multiplication of the virulent diplococci does take place, it is still likely that the toxic action is caused by the diplococci which have suffered dissolution. That this is the correct

interpretation is shown by the toxic action (1) of cultures killed by heat, and (2) of the autolyzed fluids—autolysates—of cultures. As regards the autolysates, they can readily be shown to be lethal, by intraperitoneal injection, in mice and small guinea-pigs, of which the latter are the more sensitive. The autolysate prepared by suspending the growth from a pint Blake bottle in 12 c.c. of salt solution will, after filtration through porcelain or centrifugalization till clear, cause death in twenty-four hours in doses of 0.5 to 1.0 c.c. It seems as if the filtered extracts had lost something of their toxicity as compared with the centrifugated ones. Exudates obtained from the peritoneal cavity of guinea-pigs, in which many diplococci had been dissolved, also proved to be toxic for mice. Heating the autolysate to 65° C., at which temperature the enzyme is reduced in activity, does not reduce materially the toxicity; hence, the poison is probably independent of the enzyme. The following tabulation shows the comparative effects of living and dead cultures and auto-

Weight of Pig in Grams.	Substance Injected.	Result.	Post-mortem Appearances.
187	0.5 c.c. susp. living cocci.	Died during night.	8 c.c. turbid fluid in peritoneum; typical lesions. Large numbers of diplococci; some phagocytosis.
150	0.5 c.c. susp. heated to 60° C.	Died during night.	4 c.c. haemoglobin-tinted fluid. Mottled adrenals. Oedema pancreas. No phagocytes or diplococci.
190	1.0 c.c. susp. heated to 60° C.	Survived.	
182	2.0 c.c. susp. heated to 60° C.	Survived.	
260	3.0 c.c. susp. heated to 65° C.	Died during night.	5 c.c. haemoglobin-tinted fluid; typical lesions; multiple haem. in liver; some leucocytes; few fragments; swollen cocci.
152	1.0 c.c. autolysate.	Died during night.	4 c.c. slightly turbid exudate in peritoneum; mottled adrenals; some leucocytes.
182	1.0 c.c. autolysate heated to 65° C.	Died during night.	4 c.c. clear fluid in peritoneum; red adrenals; no leucocytes in peritoneum, but some in omentum.

lysates. All the injections were made into the peritoneal cavity at four P. M.

The fact that this table especially brings out is that heating the diplococci to 60° C. or slightly above reduces their toxicity, while the toxicity of the autolysate is not materially reduced by the temperature. Upon what changes in the diplococci produced by this temperature the loss depends is not shown by this experiment, but perhaps the reduction of enzymotic power, which begins to take place about 60° C. may partially explain the effect. Since the toxic action of the diplococcus depends upon disintegration, reduction in the rapidity of dissolution might easily affect the result. Relation between the weight of the pig, as influencing susceptibility, is also shown to play a part, but one easy to be overcome by increasing the dose of the diplococcus.

In order to ascertain how important viability of the diplococcus is in bringing about the lethal effects a suspension in Ringer's fluid of which the fatal dose had been determined was kept at 2° C. until growth could no longer be obtained. It was assumed that the toxin suffers less injury when the cocci are killed at low than at the higher temperatures. The lethal dose went up from 0.1 c.c. to 0.2 c.c. of the suspension. A second observation bearing on this point was unwittingly made in the course of some experiments with immune sera. A suspension of the diplococcus in Ringer's fluid of which 0.1 c.c. was fatal in 12 to 14 hours in the preliminary test was found, the next day, to be without power to cause death in the control animals and, of course, in the immunized pigs. A large volume of the suspension was transplanted to sheep-serum agar but no growth was obtained. The assumption that multiplication of the injected diplococci takes place during the first hours after inoculation would seem to have more probability than the counter assumption that deterioration of the poison sometimes takes place rapidly from the action of unknown causes.

The most striking lesions in inoculated guinea-pigs occur in the peritoneal cavity. Unless the lethal effect is prolonged beyond one or two days, fluid exudates are obtained, and more or less pus and fibrin cover the anterior surface of the liver, and the surfaces of

the rolled-up omentum. Even with considerable purulent deposit over the liver, the fluid may be nearly clear. The pleural cavities frequently contain an excessive quantity of clear serum, and the lymphatic glands generally are swollen and congested. The main visceral lesions met with are (1) vivid congestion or hæmorrhages of the adrenal glands; (2) hæmorrhages into the mesentery, central tendon of the diaphragm and serosa of the abdominal walls and intestine; (3) gelatinous œdema of the pancreas and peri-pancreatic tissues. A detailed description of these lesions is not merited; but exception may be made of the hæmorrhages in the mesentery. This exception appears justified by the not uncommon occurrence of hæmorrhages into the skin in cerebro-spinal meningitis in man.

The hæmorrhages in the peritoneum in the guinea-pigs are distributed with much regularity. The most common places of occurrence are the mesentery of the ascending colon, below the pancreas, which has an almost circular form derived from the curved loops of intestine, the mesentery of the cæcum, and the central tendon of the diaphragm. Hæmorrhages occur less frequently in other parts of the serous membrane. The mesentery is stretched over bottle-tops, fixed in Zenker's fluid, and stained in hæmatoxylin and eosin. The preparations are thin enough to permit the examination to be made with the one-twelfth inch immersion lens. The hæmorrhages are strictly focal. They arise from capillaries and small veins; and they are common at the point of junction of several veins and capillaries. The extravasation is, usually, from one point of the vessel, but the infiltration of the vascular sheath and adjacent tissue with red corpuscles may be quite extensive. Sometimes only a few corpuscles escape. The vessels from which the blood escapes are not thrombosed; there is often no evidence of marked stasis; judging from the few polymorphonuclear leucocytes adjacent to the escaped corpuscles, inflammation is not a prominent feature. Often no change in the vascular walls at the place of escape of corpuscles can be made out; sometimes the endothelium is massed at the point of apparent escape; rarely is the vessel dilated above or below the point; but in some instances the endothelium has been displaced outwards by the escaping corpuscles.

or even appears to be deficient. The last appearance is similar to that which Flexner and Noguchi[11] described in rattle-snake venom poisoning as caused by "hæmorrhagin," an endotheliolysin contained in that venom.

It has been noted that the toxic autolysate carries all the toxic bodies contained in the diplococci. It is, however, doubtful whether all of them pass equally readily through Berkefeld filters. Larger doses of the filtered "extract" are required than of the centrifugalized ones to produce lethal results; and the exudates caused by the former may not be attended by accumulations of fibrin and pus. Possibly the small fragments of bacterial bodies, not separated by the centrifuge, bring out the richer emigration of leucocytes. Hæmorrhages have, however, been hardly ever noted among the lesions caused by the filtrate.

The great capacity which the body displays for ridding itself of the diplococci, as shown by the enormous numbers of them which disappear from the peritoneal cavity in a few hours, could be readily accounted for by the inherent tendency to self-digestion which the diplococci exhibit. Any mechanism which will either kill the micro-organisms outright, or so reduce their vitality as to bring them under the injurious influence of their own enzyme will suffice to bring about their dissolution. It has already been pointed out that phagocytosis is not essential to the removal of the diplococci from the peritoneum and the reason for this is now clear. It is relatively uncommon to find, in human infections, any number of the diplococci circulating with the blood, and in guinea-pigs, mice and monkeys, the diplococci are present in the blood for a brief relative period only. From these observations the fact might be deduced that the diplococcus does not thrive in the blood serum. David Davis[12] has observed the diplococcus to grow in defibrinated blood of certain normal individuals, but he has also ascertained that normal serum is bactericidal for the diplococcus, and this destructive property is diminished but not wholly removed by heating the serum to 60° C. for thirty minutes. Hence, the question arises whether in the guinea-pig this mechanism of serum-lysis or any

[11] *Univ. Penna. Med. Bulletin*, 1902, xv, 345.
[12] *Jour. of Infectious Diseases*, 1905, ii, 602.

allied phenomenon is employed naturally in removing the diplococci from the peritoneal cavity. An answer to this question may help to explain the bactericidal phenomena which take place in the peritoneal cavity of these animals.

I have made experiments on the dissolving power of the blood-

Two guinea-pigs received intraperitoneal injections of Diplococcus "654" heated previously to 65° C. for thirty minutes. They survived about eight hours. The clear peritoneal exudate was collected: it contained very few leucocytes and many shadowy diplococci. Centrifugalization until clear. Two series of tests were made as follows: fresh guinea-pig serum and the fresh exudate with large and small amounts of living diplococci; serum and exudate heated to 60° C. with the same amounts of living cultures. The tabulation gives the result.

SERIES I.

	Immediate Culture.	Culture after 24 Hours.	Culture after 48 Hours.
Serum 2 c.c. emulsion cocci 0.1 c.c.	Abundant growth.	Reduction in growth.	Many separate colonies.
Serum (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Idem.	Idem.
Exudate 2 c.c. emulsion cocci 0.1 c.c.	Idem.	No growth.	No growth.
Exudate (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	No growth.	No growth.
	Immediate Cover-slips.	Cover-slips after 24 Hours.	Cover-slips after 48 Hours.
Serum 2 c.c. emulsion cocci 0.1 c.c.	Many well stained cocci.	Small number of cocci remain.	Increased number of cocci.
Serum (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Reduction less than previous one; swollen cocci visible.	Greatly increased number of cocci.
Exudate 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Only shadows of cocci remain.	No cocci.
Exudate (60°) 2 c.c. emulsion cocci 0.1 c.c.	Idem.	Only detritus.	Detritus.

Series II was a repetition of Series I, except that 0.5 c.c. of the emulsion of the diplococcus was employed. The striking feature was the complete destruction of the organisms by the unheated exudate; the apparent complete destruction at the end of the first twenty-four hours by the exudate heated to 60° C. and the re-appearance of a small number of colonies from this tube at the end of the second twenty-four-hour period. The serum tubes gave abundant growth in all the transplantations. Cover-slip preparations showed reduction in numbers of diplococci in all the tubes, the reduction being less marked in the tubes of serum than of the exudates.

serum and the peritoneal exudate, from guinea-pigs on the diplococcus. The serum is collected in a sterile manner; the peritoneal exudate is freed from cells by centrifugalization. Both fluids possess digestive power for the diplococci, but in very different degrees. Mixed with fresh cultures considerable numbers of the diplococci can be destroyed and dissolved. In the case of the exudate, the toxicity, as might be expected, is increased by this digestion. The serum and exudate differ not only in respect to the number of diplococci which they destroy in their natural condition, but they also differ as regards the effect of heat on the bactericidal power. Heating to 60° C. for thirty minutes robs the serum of much more of its power to destroy the diplococci than the exudate.

Practically the same result as the preceding has been obtained with exudates derived from guinea-pigs succumbing to living cultures. The power to destroy diplococci is not lost by the exudate after several weeks keeping at 2° C. In the course of the destruction of the diplococcus by the exudate, I have repeatedly noticed the enlarged or swollen, deep-staining diplococci already mentioned of which it has been suggested that they may be more enduring forms of the micro-organism. In safranin-stained preparations these diplococci stand out as sharply defined, highly refractive, deep pink bodies. They are the last morphological elements to disappear from the fluids; and when they can no longer be detected the cultures prepared from the fluids remain, as a rule, sterile. Fresh guinea-pig serum, and to a less extent the heated serum, can destroy all the diplococci added if the number is not too great. There appears to be variations in this power in different normal animals.

The question upon what ingredients the difference in action of serum and inflammatory exudate depends, arises naturally. Perhaps the most important difference in composition would arise from the products of diplococcal disintegration and of leucocyte destruction in the exudate as compared with the serum. We have already seen that the products of diplococcal dissolution are injurious to living diplococci only when they are highly concentrated, and hence it seems as if in the case under consideration they could be left out of account. My own opinion is that the destroyed leucocytes yield to the exudate an injurious substance which acts upon the diplococci

in such a manner as to bring them under the dissolvent action of their own enzyme. Since, also, the fresh and heated (60° C.) exudate is digestive upon diplococci heated to 65° C. and 70° C., it must itself possess dissolvent power. This latter power is probably not complemental but rather proteolytic in nature. Whether the diplococci are subject in high degree to the complemental form of bacteriolysis is not known; but the power exerted by serum heated to 60° C. would rather indicate that another kind of destruction than this ordinarily takes place. What this is can be surmised from the general tendency to dissolution exhibited by the diplococcus. It is necessary only that fresh or heated serum should injure the diplococcus in order that its intracellular bacterial enzymotic power should come into action. In order to explain all the appearances met with in the peritoneal cavity of guinea-pigs infected with *Diplococcus intracellularis,* the various properties of the diplococcus, plasma, and inflammatory exudate, have to be taken into accurate account.

EXPERIMENTAL CEREBRO-SPINAL MENINGITIS IN MONKEYS.

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PLATES II–V.

At the beginning of my study of *Diplococcus intracellularis* I had the plan of using monkeys to reproduce the symptoms and lesions of cerebro-spinal meningitis in man. My study of the intraperitoneal injections of guinea-pigs encouraged me to believe that the injection of suitable cultures of the intracellularis into the spinal canal of monkeys would lead to the production of an acute inflammation, the symptoms and lesions of which might bear resemblance to those occurring in the natural infection in human subjects. I was not mistaken in this belief. Some of my earliest experiments were made with monkeys. The cultures were introduced into the spinal canal by means of lumbar puncture. The injection was made commonly in the canal at the level of the third lumbar space. This space is not below the level of the cord in many monkeys, but I have never had paralysis follow my inoculations. I endeavored to secure a flow of spinal fluid through the needle before injecting the culture, for, otherwise, doubts whether the injection was carried into the canal or the surrounding muscles might arise. Not infrequently I failed to obtain this flow of fluid even when I felt reasonably certain that the needle was in the canal. In endeavoring to remove this source of doubt I introduced, in a few instances, the needle into the fourth and fifth lumbar spaces, without causing, in a single instance, paralysis. It sometimes happened that fluid could be obtained at these higher levels when it was not secured at the lower ones. Small monkeys, as a rule, seem not to have any considerable quantity of free fluid in the spinal canal. In this respect, this class of animals differs greatly from man. The amount of fluid which is obtainable by lumbar puncture from the monkey

is subject to wide variation in the same species, and in different species. The amount which may be secured from an animal on successive days also differs greatly. In some cases, a few drops may be secured after patient waiting for five or ten minutes; in other cases, the flow sets in immediately and one or two cubic centimeters are rapidly obtained. As will appear below, the quantity of fluid is increased at certain stages of the inflammatory process, to diminish or disappear wholly at others.

In cases in which no spinal fluid has been obtained preceding the injection of the culture, a successful injection can be assumed if the monkey develops symptoms of illness within a few hours of the inoculation, or if a subsequent puncture of the spinal canal yields fluid containing the diplococci and exuded inflammatory cells. I have found that the injection of relatively large quantities of living diplococci into the muscles about the spinal column, or into the muscles and subcutaneous tissues of other parts of the body, produces only trifling effects. There appears at most some local swelling, and the animals may refuse food for a few hours. The tumefaction quickly disappears and the appetite returns. There is a parallel in this respect between the relative susceptibility of the guinea-pig to intraperitoneal inoculation and the high degree of refractoriness which it displays toward subcutaneous injections of the diplococcus. Not a few cultures of the diplococcus have failed to produce symptoms or lesions when injected into the spinal canal of monkeys. Virulence plays an important part in these injections. In some instances the degree of virulence as exhibited in monkeys and guinea-pigs was similar. Cultures which were active against guinea-pigs were active in monkeys; a culture of low virulence for the former was little pathogenic for the latter animals. This parallelism was well shown by a culture "Smith," which retained its virulence through several months of artificial cultivation. When its power to infect guinea-pigs was finally greatly diminished, it had lost to a large degree its power to cause serious lesions in monkeys. In the experiments upon monkeys to be related, suitable cultures for inoculation were, as a rule, chosen by testing them in advance upon small guinea-pigs.

At the time my experiments were begun there was, as far as I

was aware, only one report in the literature upon the use of monkeys to produce cerebro-spinal meningitis by injecting cultures of *Diplococcus intracellularis* into the central nervous system. Bettencourt and Franca[1] attempted without success to infect monkeys. They trephined one monkey and introduced the culture beneath the dura mater; they injected in two other monkeys cultures into the spinal canal. In still another monkey they attempted to infect the animal by rubbing the nasal mucous membrane with cotton moistened with a culture of the intracellularis. The successful experiments of Von Lingelsheim and Leuchs[2] were published after my first communication on this subject had appeared.[3] Weichselbaum[4] succeeded by subdural inoculation in three instances in dogs in producing acute pachy- and leptomeningitis associated with encephalitis; Councilman, Mallory and Wright[5] successfully produced acute meningitis in a goat by injecting a bouillon suspension of the diplococcus into the spinal canal. Albrecht and Ghon,[6] and Bettencourt and Franca[7] failed to confirm this result in their experiments upon goats.

The monkey offered itself as a suitable animal for the inoculation experiments because of its relationship to man; the fact of its upright carriage, which would permit comparison with man in respect to the distribution of the lesions, and because of the ease and safety with which repeated lumbar puncture could be made in following the course of the disease. The protocols of certain successful experiments will be given.

Monkey No. 1. Macacus Nemestrinus.—April 12, 1905, 10:30 A. M. A moderately large monkey was etherized,[8] after which a needle was introduced into

[1] Uber die Meningitis Cerebro-spinalis Epidemica und ihren specifischen Erreger. *Zeit. f. Hyg. u. Infectionskrank.*, 1904, xlvi, 463.

[2] Tierversache mit dem Diplococcus intracellularis (Meinigococcus), *Klin. Jahrbuch*, 1906, xv, 489.

[3] Experimental Cerebro-spinal Meningitis and its Serum Treatment, *Jour. Amer. Med. Assoc.*, 1906, xlvii, 560.

[4] *Fortschritte der Medicin*, 1887, v, 622.

[5] Epidemic Cerebro-spinal Meningitis and its relation to other forms of Meningitis, Boston, 1898.

[6] *Wien. klin. Woch.*, 1901, xiv, 984.

[7] *Op. cit.*, page 500.

[8] In later experiments ether was not used, as the operation gave little pain.

the spinal canal. Clear fluid escaped from the needle. A culture eighteen hours old of *Diplococcus intracellularis*, in its first generations on Loeffler's serum, suspended in salt solution, was injected. The monkey quickly recovered from the operation. Six hours later it appeared to be sick; at 6 P. M., it no longer sat on its perch; 9 P. M., still on bottom of cage, very sick, head down. On being disturbed, it moved slowly. Death during the night. Survived injection probably eighteen to twenty hours.

Autopsy 9 A. M., April 13. The internal organs, except the nervous system, show no striking lesions. Brain and Spinal Cord.—No marked excess of fluid in the meninges. The convex surface of the brain is greatly congested, and punctiform hæmorrhages exist in the pia-arachnoid and the superficial portions of the cortex. No definite exudate can be made out on the convex surface of the convolutions, although the pia of the sulci appears white and is slightly thickened (probably an old condition). A focus of softening or an abscess, the size of a large pea, occurs to the right of the superior longitudinal sinus in the convolutions anterior to the motor area, and just beneath the membranes. The base of the brain is covered with an opaque exudate, white in color, which extends over the medulla and, anteriorly, to the optic commissure. The lumbar and thoracic portions of the spinal cord are covered, chiefly posteriorly, with an opaque white exudate. The cerebral ventricles contain an increased amount of fluid of turbid appearance.

The diplococcus was recovered in cultures from the exudates of the spinal cord and base of the brain, and from the blood of the heart. Cover-glass preparations from the exudates show that (1) the pia of the convexity contains many polymorphonuclear leucocytes and few diplococci; (2) the pia covering the medulla contains large numbers of leucocytes and intracellular cocci, and a small number of extra-cellular cocci; (3) the ventricles contain desquamated ependymal epithelium, many leucocytes, and few diplococci; and (4) the focal lesion in the convexity of the brain consists of a collection of polymorphonuclear leucocytes containing very large numbers of typical diplococci. Few or no diplococci occur outside of cells in this focus (Plate II, Fig. 1).

Sections of the brain and cord show an abundant emigration of polymorphonuclear leucocytes into the leptomeninges chiefly. The pia-arachnoid of the convexity contains a richer exudate than was evident to the naked eye; but the exudate covering the base of the brain is present in much the greater amount. It consists chiefly of leucocytes; fibrin occurs in very fine strands and in small amount only. The ventricular fluid contains many leucocytes and no fibrin. A striking feature of the inflammation is the invasion from the pia-arachnoid of the superficial portion of the cortex directly, and of removed parts at considerable depths indirectly along the sheaths of the blood vessels. The vascular sheaths and the perivascular lymphatic spaces are richly infiltrated with polymorphonuclear leucocytes from which locations the surrounding brain tissue is being invaded by emigrating leucocytes. Sections through the filum terminale show the leucocytes to surround the cord in the pia, a slight invasion along the septa into the nervous tissue, and thick perivascular emigration about the deepest vessels of the dura mater.

This successful experiments was obtained by the use of a recent

culture isolated from the spinal fluid of a child "Kepp." The fluid was quite opaque from the many leucocytes present and it contained a moderate number of the diplococcus, both within leucocytes and free in the fluid. A second monkey of the same species and about the same size as the first was inoculated with one cubic centimeter of the sedimented exudate. Immediately after the injection, the monkey became rigid and the head was retracted; recovery took place in a short time. In spite of the fact that no fluid escaped from the needle, no doubt was entertained that the injection entered the spinal canal. However, no symptoms of disease developed from this injection.

Monkey No. 3. Small Macacus rhesus.—April 15, 1905, 9. A. M. Inoculated by lumbar puncture with two loops suspended in salt solution of "Bingley" culture of the diplococcus from spinal fluid. 5 P. M., animal sick; sits on bottom of cage. 9 P. M., very sick; crouches on bottom of cage with head depressed; moves slowly on being disturbed. April 16, 9 A. M., still alive; head down almost to level of the floor; a little later, the monkey is lying on one side and is passing through a convulsion. The convulsive seizures follow each other at short intervals and are excited by sudden noise or by contact. 3 P. M., convulsions still occurring; lumbar puncture yields a small quantity of bloody fluid which on microscopical examination shows many polynuclear leucocytes containing typical diplococci. 10 P. M., convulsions continue. Animal died during the night; probably survived about forty-three hours.

Autopsy, 9 A. M., April 17. No visible lesions of the internal organs except the central nervous system. The convex surface of the brain shows great injection of the pial vessels; and the pia-arachnoid contains a gelatinous fluid exudate. Purulent exudate is visible surrounding the infundibulum only. The fluid in the ventricles is slightly increased. No visible exudate covers the spinal cord. The cultures made from different portions of the brain and cord remained sterile. Sections of the spinal cord show a small quantity of purulent exudate in the meninges; those from the brain show a richer leucocytic infiltration of the pia-arachnoid, and the invasion of the choroid plexus of the lateral ventricles. The meninges of the sulci are especially infiltrated. From the superficial meninges, and from the intra-cortical vessels, the brain substance has been infiltrated with many leucocytes.

As regards this experiment I wish to point out that when the inoculated monkey survives through the second day, the quantity of exudate may not be very considerable, and the diplococci, if still present, may fail to grow on a suitable culture medium.

Monkey No. 7. South American; Genus Cebus.—May 19, 1905, 3 P. M. One cubic centimeter of a suspension of spinal fluid culture "Goldman" injected. This South American species is considerably smaller than the Macacus monkeys employed. It was found dead at 7 A. M., May 20, but evidently had been dead

only a short time. Probably survived fifteen hours. The autopsy showed a small amount of exudate over the spinal cord in the lumbar region, turbid fluid along the base of the brain to the optic commissure, vivid injection of the pia of the convexity, and an increased quantity of turbid fluid in the cerebral ventricles. Cultures were positive. Smear preparations from different parts of the brain and cord show the leucocytic exudate to be general, and a fairly large number of diplococci, chiefly intracellular, to be present. Many of the diplococci within leucocytes are swollen or otherwise degenerating. The preparations made by smearing bits of the nasal mucosa on slides show a considerable number of polymorphonuclear leucocytes among the high epithelium and mucus. *A small number of the leucocytes contain many diplococci morphologically like those of the exudate in the brain, and presenting the same degenerations.*

A study of the brain and cord in sections shows the inflammatory lesions over the convexity to be more pronounced than was evident to the naked eye. The pia is everywhere invaded by polynuclear cells, and the exudations form thick, wedge-shaped infiltrations between the convolutions. A fine network of fibrin unites the leucocytes. The invasion of the cortex is a marked feature of the lesions. The leucocytes have passed into the brain tissue from the surface and along the sheaths of the vessels (Plate II, Fig. 2). An intermediate zone of non-infiltrated tissue exists between the two layers. The invasion of leucocytes into the brain stops rather abruptly a centimeter or so from the surface, and the deeper vascular sheaths are devoid of these cells. Among the leucocytes which have emigrated into the cerebral tissue are a small number of eosinophilic cells. A section through the medulla shows less exudate than some parts of the cortex. The deeper parts of the medulla have also been invaded from the vascular sheaths. The lateral ventricle has been invaded along the choroid plexus, and an accumulation of leucocytes occurs below the epithelium, which is partly deficient, at one side of the ventricle. The thoracic region of the spinal cord is relatively free from exudate.

A spinal root ganglion included in a section of the spinal cord is surrounded, beneath its fibrous capsule, by a collar of leucocytes which are penetrating among the nerve cells (Plate III, Fig. 3). Sections of the optic nerve show leucocytic invasion. Diplococci are numerous in the leucocytes in the pia-arachnoid. They cannot be found with certainty in the leucocytes or free in the brain tissue.

This experiment serves to show that in a brief period of fourteen or fifteen hours advanced and deep lesions can be produced in the brain and its membranes by *Diplococcus intracellularis*. The experiment also indicates that the inflammatory reaction may be more active in the brain than in the spinal cord. The inflammatory exudate resembles in character that present in acute cases of epidemic meningitis in human subjects. Emphasis should, perhaps, be laid upon the findings in the nose and in the spinal ganglion. It is usual to find in the monkeys succumbing to the experimental infections, evidences of inflammation of the nasal mucosa. Smear preparations from the superior mucous membrane often show a

variable number of leucocytes enclosing diplococci presenting the morphology and staining properties and the degenerations of the diplococci in the brain and cord. The inflammation and purulent infiltration of the spinal ganglion is interesting in relation to the similar finding in cases of cerebro-spinal meningitis in man by Councilman, Mallory and Wright.[9]

Monkey No. 10. Macacus rhesus.—May 26, 1905. At 11 A. M. one half agar culture "Cohn" suspended in salt solution was injected into the spinal canal. Fluid escaped from the inserted needle. 5 P. M., animal very sick; living at 9 P. M. Found dead at 6 A. M., May 27. Probably survived fifteen hours.

Autopsy: The meninges of the inoculated portion of the cord show minute hæmorrhages and cloudy exudate. The meninges of the medulla contain an increased quantity of cloudy fluid. The pia covering the convex surface of the brain is hyperæmic, especially over the occipital lobes, which present an almost uniformly reddish tint; while the pia and the adjacent brain substance are everywhere beset with minute hæmorrhages. Cultures from the lumbar portion of the cord, third ventricle, and pia over the medulla all give pure growths of the diplococcus. No growths are obtained from the cortical meninges and the lateral ventricle. The furthermost portion of the dura mater, extending beyond the olfactory lobes into the nose, is covered with an inflammatory exudate in which polymorphonuclear leucocytes carrying biscuit shaped Gram-negative diplococci exactly resembling in form, preservation and staining, those present in the meninges of the brain and cord occur. Smear-preparations from the turbinated bones show very few leucocytes and no diplococci. Cultures made from the mucus of the turbinated mucous membrane did not yield the diplococcus.

The sections of the brain show a moderate inflammatory œdema affecting the pia-arachnoid of the base and the convexity. The especial pathological conditions to be emphasized are (*a*) the great congestion of the pial blood-vessels and the extravasation of red corpuscles; (*b*) the direct inflammatory invasion of the superficial cortex from the pia which is associated with rarification of the brain tissue; (*c*) the profound changes in the blood-vessels of the brain proper and the degeneration and leucocytic invasion of the surrounding brain tissue (Plate III, Fig. 4). In some parts of the cortex, adjacent to the sulci, the leucocytic accumulation is so rich as to simulate abscess formation; no softening has, however, taken place. The emigration of leucocytes from the intracortical vessels was active at the time of fixation of the tissues. The perivascular lymphatics contain fibrin and coalesced red-corpuscles, and the blood-vessels themselves are occluded, in places, by coalesced (agglutinated) red corpuscular and by fibrinous thrombi. The punctiform hemorrhages in the brain tissue arise from these injured and occluded vessels. *Sections of the dura mater extending beyond the olfactory lobes show a richer accumulation of leucocytes than elsewhere in the membrane itself and an invasion of the substance of the membrane.*

Typical diplococci occur within leucocytes in the meningeal exudation and

[9] *Op. cit.*, p. 114.

they are moderately numerous. They exist also in the leucocytes in the brain tissue, adjacent to the blood-vessels from which the leucocytes have emigrated. No diplococci are found in the brain tissue outside of cells.

A lengthy discussion of this experiment is not called for since it carries its own explanation. Attention may, perhaps, be directed again to the co-incident effects produced by the diplococcus upon the membranes and the nervous tissues. The wide involvement of the blood vessels is a significant fact in the pathology of this experimental disease; and the evidence of diffusion of the poison from the blood vessels of the brain or from the peri-vascular lymphatics into the brain tissue is made strong by this experiment. The agglutinative red corpuscular thrombi constitute a novel feature of the pathological condition. The rich assemblage of leucocytes surrounding the olfactory lobes and extending into the substance of the dura mater seems to me not wholly without significance in view of the possibility of infection of the nasal passages with diplococci from the brain.

In view of the importance of this condition for explaining the appearance at times, of *Diplococcus intracellularis* in the nasal and pharyngeal cavities in human subjects of cerebro-spinal meningitis, I gave especial attention to the study of the olfactory nerves and the nasal passages in several monkeys. It may, in the first place, be stated that the turbinate and septal mucous membrane will be found to be vividly congested in all the animals which succumb to the acute infection. If smear preparations from the mucous covering the inflamed membranes, or, better, from bits of the mucous membrane snipped off with scissors be examined, a variable but increased number of polynuclear leucocytes will be seen. If the leucocytes are compared with those present in the meninges about the base of the brain, they will be found in the same condition of preservation or degeneration as the latter. Moreover, in those cases in which diplococci are still demonstrable in the leucocytes in the brain, a certain number, sometimes relatively many, of the leucocytes in the nose contain Gram-negative diplococci resembling in all external features the former micro-organisms. If the dura mater beginning at the olfactory bulbs, surrounding the olfactory nerves, and extending through the cribriform plate into the nose, be carefully removed with the adjacent

portion of the ethmoid bone and olfactory mucous membrane, fixed in Zenker's fluid, sectioned longitudinally, stained in hæmatoxylin or methylene blue and eosin, and examined microscopically, the passage of leucocytes from the brain cavity into this membrane and about the olfactory nerves towards the ethmoid, can be traced. The blood-vessels of the adjacent olfactory mucous membrane are seen to be dilated and leucocytes to be passing into the layer of columnar epithelium of the surface.

The abundant lymphatics of the mucous membrane are in communication with the lymphatic spaces which enclose the branches of the olfactory nerves, and these spaces again communicate with the subdural and subarachnoid spaces of the cranium, so that the lymphatics of the nasal mucous membrane can be injected, according to Schwalbe and Key and Retzius, from the cranial cavity.

I have not succeeded, although I have made several attempts, in cultivating *Diplococcus intracellularis* from the nasal mucosa of these monkeys. I realize, of course, that it is highly desirable and very important to bring this final proof of the passage of the diplococci from the cranial cavity into the nose. The difficulties which surround the demonstration are considerable, for I think it probable that the diplococci do not long remain viable in the nose in monkeys. I conceive the conditions there to be, possibly, even more unfavorable to them than in the meninges where, indeed, they tend rather to disintegrate than to multiply. The conditions in man are, of course, very different and far more favorable to the existance of the diplococci. But in spite of the incompleteness of the proof in the case of the monkeys, I would still urge that attention be given to this possible mode of infection of the nasal and pharyngeal passages in human subjects.

The next experiment to be described brings out a different point, namely, that of a fatal issue, after the lapse of thirty hours, with which is associated the gradual diminution and degeneration of the diplococcus, without any marked participation of leucocytes in the process, or production of striking pathological lesions. It recalls forcibly certain observations made on guinea-pigs.

Monkey No. 12. South American; Genus Cebus.—June 1, 1905, at 11 A. M., a small monkey was inoculated with one half agar slant culture "Smith"; fluid

flowed from the needle before injection. 12 M., lumbar puncture (=l. p.) 1 c.c. almost pure blood obtained; it showed on cover-slips (=c. s.) many free diplococci. 3:15 P. M., l. p. small quantity blood secured; c. s. many cocci free and very few in leucocytes. Animal not sick. 9 P. M., l. p. a few drops of slightly blood-tinted fluid obtained; c. s. many free diplococci and a small, although increased number of leucocytes containing diplococci. Animal less lively than before. June 2, 9 A. M., monkey is very sick; temperature subnormal; head droops below arms; no convulsions on disturbance; l. p. a few drops of blood-tinted fluid secured; c. s. leucocytes increased, some containing diplococci part of which stain feebly; many free diplococci. 3 P. M., l. p. leucocytes still not numerous; intracellular and extracellular diplococci present, but the number is diminishing. Cultures from the fluids obtained at the punctures gave growths in every instance. About 4 P. M. the monkey showed general weakness and retraction of the head. Dr. Meltzer, who examined the animal, pronounced the latter condition not to be opisthotonus. Death at 5 P. M. On ice till next day.

Autopsy, June 3, 9 A. M. Neither the cord nor the brain show any marked lesions.

The pia of the cord is slightly injected; a small quantity of clear fluid is present in the pia of the medulla; the intraventricular fluid may be slightly increased. Cultures are negative from brain and cord and the heart.

Smear preparations from the meninges of cord and brain and the fluid of the ventricles show a small or moderate number of diplococci lying free, and almost no leucocytes whatever except in the spinal cord at the point of inoculation. A moderate number of the cells containing diplococci, many of which are degenerating, occur there. Elsewhere it is exceptional to find polynuclear cells containing diplococci. The failure of the diplococcus to grow in cultures made at autopsy does not signify, since the power to grow may be quickly lost at the temperature of the refrigerator. Smear preparations from the nose show no typical diplococci, while a few typical organisms are present in the smears from the pia about the olfactory lobes. The microscopical examination of sections of the spinal cord and brain bear out the naked eye appearances. The membranes show a slight but definite accumulation of leucocytes; there is no œdema, and the nervous tissue is free from appearances of degeneration or from leucocytic invasion. The leucocytes are, however, increased in numbers in the cortical vessels.

The next experiment records an instance of fatal effects, from a relatively small dose of the diplococcus, which was delayed until about sixty hours after injection. The gross lesions were slight; the microscopical ones were definite and interesting because the period of infection was long enough to permit of reaction on part of the fixed tissue cells.

Monkey No. 18. Macacus rhesus.—June 24, 1905, 10 A. M., injected one third culture "Smith." Symptoms of illness developed in the usual time and were pronounced during the first twenty-four-hours. June 25, 12 M., lumbar puncture. The fluid obtained showed many leucocytes, but no diplococci. Cul-

ture negative. The animal did not fully recover and died about sixty hours after inoculation. The autopsy revealed congestion of the meninges, and a small excess of turbid fluid in the lateral ventricles. Cultures on sheep-serum agar were made from several parts of the nervous system. Growths were obtained on the second day, starting from the condensation water, in the tubes inoculated from the third ventricle and lumbar cord. Sections from the brain and cord show, first, a marked congestion of the veins which is greater in the meninges of the brain than of the cord. Second, a small degree of leucocytic exudation throughout the meninges, which in the brain is more abundant in the sulci than over the convolutions. But the most striking and, perhaps, important feature is the great increase of large monoculear pial or connective-tissue cells which by reason of their number make up a large part of the exudate. These cells are larger than leucocytes, possess single vesicular nuclei excentrically placed, and pale and transparent protoplasm.

To be contrasted with the previous experiment is the next one in which the inoculated monkey survived hardly more than twelve hours, in the course of which period marked gross lesions appeared in the nervous system.

Monkey No. 17. South American; Genus Cebus.—June 22, 1905, 10 A. M., injected one half agar culture "Smith," twenty-four hours old. Fluid flowed from needle before injection. 9 P. M., monkey dying. Found dead at 6 A. M., June 23. The autopsy showed the entire spinal cord congested and infiltrated with turbid fluid. In the cerebral meninges smaller and larger hæmorrhagic foci were visible. The pia of the pons and medulla was vividly congested, and a fluid exudate occupied the meshes of the pia-arachnoid generally, and was most abundant over the medulla. The ventricles were unchanged, apparently. Cultures gave the following result: Abundant growth of the diplococcus from the lumbar cord and base of brain; a few discrete colonies from the cortex; no growth from the lateral ventricle. Smear preparations bore out the naked eye indications of the distribution of exudate and diplococci. Sections of the tissues show marked leucocytic infiltration of the membranes of the cord and brain, and a greater accumulation of cells in the basal membranes and the membranes of the sulci. The infiltration is wholly polymorpho-leucocytic. The most striking lesion, however, is the hæmorrhage which is focalized in the membranes and in the superficial brain matter—in the basal and cortical parts. The hæmorrhages in the brain tissue proper have arisen (*a*) by direct extension from the menigeal extravasations, and (*b*) from the vascular branches within the substance of the organ itself. They form elongated or circular foci, depending on the direction of the section. Another lesion of interest is an acute endarteritis which affects the larger and smaller arteries, chiefly at the base of the brain. A sub-intimal infiltration of cells consisting of polymorphonuclear leucocytes and mono-nuclear cells is seen. The vessels are rarely wholly or almost occluded by this accumulation of cells beneath the intact endothelium. Acute encephalitis was a prominent feature of this case.

This experiment, taken into account with others to be described, tends to show that the diplococci rise in the spinal canal to the

medulla, spread themselves over the base of the brain, extend into the cortical meninges, and lastly enter the ventricles. The inflammatory lesions follow in the wake of this extension and doubtless are directly due to the presence of the diplococcus. Attention should perhaps be directed to the hæmorrhages; and to the acute endarteritis mentioned in this protocol of which other examples will be described.

Monkey No. 25. Macacus rhesus.—June 21, 1906, 11 A. M. Inoculated with one agar culture "596." Fluid flowed from needle before injection. 2 P. M., animal appeared sick; lay on bottom of cage, but on being disturbed sat up. 6 P. M., depression increased. 12 P. M., still lying on bottom of cage, but not prone and rises to sitting posture when disturbed. June 28, 8 A. M., brighter; sits up. 12 M., attempted lumbar puncture. Animal resisted with considerable vigor making the puncture difficult. The struggles brought on sudden collapse from which partial recovery took place. Monkey lay on bottom of cage breathing very rapidly; drank water. 2 P. M., still very sick. From this time until 3:45 P. M., when death occurred the animal became weaker and developed intermittent convulsions. The autopsy showed no general visceral lesions. On exposing the spinal cord, it was found congested and covered with a thin, whitish exudate which was more abundant in the lumbar region than in the thoracic and cervical regions. The blood-vessels of the dura mater were injected; but the turgid pia vessels showed through this membrane. On removing the dura, the pia-arachnoid was seen to be vividly injected, the congestion affecting the main and smaller vascular branches and producing a remarkable picture of hyperæmia. There was no visible exudate over the convex surface of the brain. At the base, extending from medulla to optic commissure, there was a turbid fluid exudate. The ventricles contained turbid fluid, but were not dilated. The basal exudate followed the dura over the olfactory lobes into the ethmoid plate. The nasal mucosa was congested. Cover-slips showed the following: From the lumbar spinal cord many leucocytes and diplococci, the latter both free and in cells; from the thoracic and cervical regions of the cord, many leucocytes and fewer cocci; from the convexity—anterior and posterior—impression preparations indicated that few or no leucocytes collected in this region, and the diplococcus was almost wholly absent; from the medulla many leucocytes and extra- and intracellular diplococci; from the ventricles leucocytes and diplococci in small numbers; from the deepest part of the dura at the ethmoid bone many polynuclear leucocytes, but no typical examples of the diplococcus. Cultures from all the sources remained sterile. Since more than two days elapsed, owing to accidental circumstances, between the death of the monkey and the autopsy, it may well be that the failure of cultures was due to this cause. The same condition is capable of diminishing the number of diplococci which can be made out in smear-preparations. But the distribution of emigrated leucocytes showed clearly where the infection was severe, and where it was in its beginning stages. The sections of the tissues bear out the gross findings. They show the existence of great congestion of the pial veins, a small degree of leucocytic infiltration of the pia covering the con-

volutions, and somewhat greater accumulation of inflammatory cells in the sulci. The pia of the deeper parts of the sulci is hardly more than œdematous. The brain substance shows no leucocytic invasion, and the veins do not carry any marked excess of leucocytes. The lower spinal cord shows rather more emigration of leucocytes than the brain. The exudate is collected on the surface of the cord, upon and between the nerve roots (Plate IV, Fig. 5). Fibrin occurs as fine strands and in small quantity.

This experiment brings out several points, some of which have not been especially emphasized before. The effects of muscular exertion on the progress and termination of the infection should, perhaps, be remarked. Since in no other experiment was so great a degree of vascular congestion noted, I should be inclined to view this condition as one of the striking consequences of the muscular over-exertion. It is a short step from this assumption to supposing the marked œdema of the pia in the sulci to have followed the congestion. This view is rather borne out by the poverty of the œdematous fluid in emigrated cells. It has been pointed out on the basis of some of the earlier experiments, and the same fact is indicated by this experiment, that the injected diplococci tend, regularly, to be distributed upwards along the spinal cord and the base of the brain. The invasion of the pia of the convex and mesial surfaces of the brain takes place at a later period, and the infection of the ventricles also takes place later, either because the diplococci are delayed in reaching them, or because they resist infection longer. There is, moreover, relation between localization of the diplococci and the degree and extent of the exudation. Where the accumulation of inflammatory products is most marked the diplococci will, if still present at all, be most abundant. Finally, the growth of the diplococcus upon a suitable culture medium will be greatly influenced, if not wholly determined, by the conditions to which the infected body is subjected after death. While a temperature just above the freezing point will preserve the morphological elements of the body and even the body of the diplococcus, yet if it is maintained for many hours, it may render the diplococcus incapable of multiplication. Higher temperatures subject the body not only to autolytic and putrefactive processes which interfere with the pathological examination, but they deprive even more quickly than cold the diplococcus of power of growth and, under certain conditions, of staining properties as well.

The temperature at which the body is kept, and the elapsed period of time between death and the examination, have a direct bearing upon the cultivation of *Diplococcus intracellularis* from the infected body. I am of the opinion that these factors operate in much the same way in cases of human meningitis as in the case of the artificially infected monkeys and guinea-pigs. Certain discrepancies in the bacteriological studies of cases of meningitis in man may, in view of these considerations, receive an explanation.[10]

On account of its bearing upon this subject and as a transition to the next topic to be discussed, an experiment in which the influence of two extremes of temperature upon the diplococcus was tested, will be introduced here.

Monkey No. 36. Macacus rhesus.—October 24, 1906, 3:30 P. M. After clear fluid was obtained, one cubic centimeter of a turbid suspension of Culture "654" was injected. The injected quantity was the equivalent of two agar slants; the culture had proved itself of relatively low virulence for guinea-pigs. October 25, 10 A. M., monkey sick; sits on perch holding head in hands; easily disturbed. L. p. no fluid obtained; needle-point covered with exudate consisting of leucocytes crowded with diplococci staining sharply. The nuclei of a small number of cells contained cocci. October 26, 12 M., monkey brighter and more active. L. p. slightly cloudy fluid flowed at once from the needle. C. s. showed leucocytes in good numbers, some of which contained few and others very large numbers of cocci staining feebly. Many of the leucocytes had probably recently emigrated since they were of normal appearance with circular (horse-shoe), non-fragmented nuclei. October 27, monkey recovered. November 10, animal in excellent condition.

November 14, 4 P. M., l. p. gave fluid readily; it flowed from needle as if under some pressure. One cubic centimeter of suspension of coccus "596" injected. No immediate effect produced. 6 P. M., monkey less lively. November 15, 7 A. M., found dead; evidently died only a short time before. Autopsy. The spinal membranes were distended and on incision a small quantity of turbid fluid escaped. The cord appeared pale, probably because of the turbid exudate which covered it. The convex surfaces of the brain were moist; definite inflammatory exudate was not visible; the basal membranes, from the medulla to the optic commissure, were infiltrated and distended with a turbid exudate. The pituitary body was surrounded with exudate; the ventricles were not widely dilated. The nasal mucosa was pale. Of the other organs only the lungs showed a pathological condition, namely, congestion and œdema. Cultures from the brain and cord, made at 4 P. M., gave growths of the diplococcus; the heart and lungs gave no growth. At 5 P. M. two segments of the medulla, about 0.5 centimeter thick,

[10] Westenhoeffer, Pathologisch-anatomische Ergebnisse der oberschlischen Genickstarreepidemie von 1905, *Klinisches Jahrbuch*, 1906, xv, 447.

were placed in sterile glass dishes which were enclosed in second dishes filled with cotton saturated with water. One was put at 37° C., the other at 2° C. At 10 A. M. next day, cover-slips and cultures were made; the latter gave no growth. C. s. showed the diplococci to have disappeared almost entirely from the tissue kept in the thermostat, the few remaining cocci being pale and ill-staining; and the diplococci from the second piece of tissue kept at 2° C. to be, perhaps, somewhat less numerous than in the control, but to stain sharply and well.

Histological examination of the central nervous system establishes the existence of a severe acute fibrino-purulent inflammation of the meninges of the cord and brain. The exudate in the cord is thicker over the posterior surface and it surrounds all the nerve roots; the dura mater is also invaded with pus cells. The exudate covering the medulla is abundant, while that covering the cortical surfaces is slighter in amount. The sulci show more exudate than the convexities. Two appearances should be emphasized: the great proliferation of endothelial cells of the pia, which cells mingle with the polynuclear leucocytes; and the diplococci, which are abundant in the exudate of the cord and medulla, innumerable in the exudate about the pituitary body and sparse in the exudate of the cortex and ventricles. The diplococci are, excepting those about the pituitary body, almost exclusively intracellular; and although a slight grade of encephalitis occurs, very few diplococci can be discovered in the leucocytes in the brain tissue. Thrombi of leucocytes occlude many veins.

The experiments reported thus far would seem to establish conclusively that the lesions and, to a certain extent, the symptoms of acute meningitis as they occur in man can be reproduced experimentally in monkeys. Since in the course of the experiments, the symptoms during life were observed incidentally only, the striking ones alone were recorded. Attention should be directed to the occurrence of nystagmus in a rhesus monkey which succumbed to inoculation. It should be stated, also, that not all the monkeys inoculated into the spinal canal succumb to the injections, next that after apparent recovery death may take place suddenly from causes which the post-mortem examination may fail to reveal, and finally that subcutaneous and intramuscular injections of large quantities of the diplococcus cause slight or no evident symptoms of disease. The cases of recovery will be illustrated by a few protocols. It should be noted that as recovery proceeds spinal puncture may show increasing cellular exudation into the canal and progressive degeneration of the diplococci. Cultures from the exudate at first positive become negative before all the diplococci disappear from it.

Monkey No. 5. Macacus nemestrinus.—May 15, 1905. This animal had been injected, April 12, 1905, with a purulent exudate without result (page 146). At

4:30 P. M. one cubic centimeter of a turbid suspension in salt solution of a diplococcus in its fifth generation from the spinal canal of case "Behren" was injected into the spinal canal. No symptoms had appeared by 9:30 P. M. May 16, 8 A. M., animal refused food and appeared sick. 11 A. M., had left perch and sat on bottom of cage. Appeared unable to lift himself on to the perch; while being observed two convulsions occured. Placed on the perch, he held on with hands and feet and was in a state of unstable equilibrium. Made no effort to escape from the cage on being handled. 10 A. M., lumbar puncture; a small amount of bloody fluid containing a moderate number of leucocytes and extra- and intracellular diplococci obtained. 3:30 P. M., l. p.: a small quantity of purulent fluid secured. The greater number of cocci were within leucocytes, many of which showed fragmented nuclei. Cultures from the exudate positive for the diplococcus. 6 P. M., back on perch. May 17, 8 A. M., again on bottom of cage; looked very sick, but took a little milk. 10 A. M., l. p.: the number of diplococci had diminished; very few were outside of cells; those in leucocytes stained feebly. May 18, 10 A. M., l. p.: a few drops of blood-tinted fluid secured; c. s.: some polymorphonuclear leucocytes, increasing number of mononuclear leucocytes, and a very small number of leucocytes containing degenerated diplococci; a few extracellular diplococci still present. Cultures negative. This monkey recovered rapidly.

Monkeys No. 19 and 20. Macacus rhesus.—June 27, 1905, 10 A. M. Each of these monkeys was injected with one third culture "Smith." They became very sick during the afternoon, and lay on the bottom of the cage. 10 P. M., still very sick. June 28, 9 A. M., both animals looked brighter. 10 P. M., No. 20 seemed livelier than No. 19. June 28, 12 M., l. p.: both yielded a small quantity of turbid fluid. The fluid from No. 19 and No. 20 contained many leucocytes; the fluid from No. 19 contained typical intracellular diplococci in small numbers, while that from No. 20 contained none. A culture was made from each fluid: No. 19 gave one colony of the diplococcus, No. 20 gave none. Complete recovery quickly followed in both instances.

Monkey No. 35. Macacus rhesus.—July 10, 1906, 10 A. M. Given 1 c.c. of thin suspension of Diplococcus "596." Spinal fluid obtained with syringe. The only symptoms which developed were slight depression, and erection of the hair covering the body. July 11, 10 A. M., l. p.: a small amount of whitish fluid from which the cells quickly subsided, was obtained. C. s. showed a large number of agglomerated, polymorphonuclear leucocytes, and very few diplococci, chiefly contained in the cells. The few free cocci could easily have come from broken-up leucocytes. At this time, a second injection of 0.5 c.c. of a similar suspension of Diplococcus "596" was made; no result. Animal quickly recovered.

To insure a fatal outcome a diplococcus of known virulence in a sufficient dose must be injected. Individual differences in susceptibility occurred among the monkeys, but no monkey was wholly refractory to inoculation. As compared with the doses which probably determine infection in man those used to produce the experimental disease in monkeys are colossal. As an example of

refractoriness the next experiment will serve; it will show also how small the effect produced by subcutaneous and intramuscular injections of the diplococcus may be.

Monkey No. 26. Macacus rhesus.—October 28, 1905, 12:30 P. M. One "Smith" culture injected. 3:30 P. M., l. p.: turbid fluid flowed from the needle. C. s. showed many diplococci and very few leucocytes. Some of the cocci grouped about the leucocytes. October 29, 12 M., l. p. not successful; one half culture "Smith" injected (probably into muscles of back). October 30, 3:30 P. M., l. p. unsuccessful. Injected two cultures "Smith" (probably into muscles of the back). 6 P. M., animal slightly depressed; off perch. October 31, A. M., animal active. 3 P. M., l. p. yielded with difficulty a small amount of bloody fluid containing leucocytes and few diplococci. Injected three cultures "Smith"; no effect. This monkey showed remarkably little depression following the injections, and was soon in a normal condition.

It is somewhat remarkable that the later injections failed to influence the result of the first injection, which evidently reached the spinal canal. I have observed many other examples of the relative innocuousness of the diplococcus when introduced beneath the skin and into the muscles. The effects of large doses given in this way may be nil, or a slight elevation of temperature may appear and remain for a few hours, or the animal show for a brief period disinclination for food. The next experiment is introduced as an example of sudden death, after apparent recovery from an inoculation.

Monkey No. 21. Macacus rhesus.—July 1, 1905, 10 A. M. Two thirds culture "Smith" injected. No fluid obtained prior to injection. No symptoms developed, but the canal must have been entered as lumbar puncture July 2, 12 M., yielded a small quantity of turbid fluid containing leucocytes and very few degenerating, intracellular diplococci. No growth was obtained on sheep-serum agar. No symptoms of disease were noted in this animal. July 6, found dead. At the autopsy more clear fluid than is usual escaped from the dura surrounding the cord. The lower segment of the cord was congested. No exudate was visible and the ventricles were not dilated. The pial vessels of the medulla were injected. Smear preparations from the spinal cord and several parts of the brain showed neither diplococci nor polymorphonuclear cells. The sections of the tissue show no inflammation or leucocytic infiltration except superficially in the lumbar region in the posterior columns of the spinal cord at what was probably the point of entrance of the needle.

In contrast to the ease with which the acute inflammations can be excited is the difficulty which attends the production in monkeys of a subacute form of meningitis. The intraspinal injections pro-

duce either an acutely fatal meningitis, or an acute disease from which recovery takes place rapidly. The animals which survive inoculation are usually well of the disease in three or four days. In rare instances, the animal lingers a victim of the disease for a longer period than this to succumb in the end. Monkeys which survive the second day after inoculation tend rather to recover than to die. By following in inoculated monkeys the changes in the cerebro-spinal canal by means of lumbar puncture, the progress of the infection can be traced and the result often predicted. Disappearance of the diplococci early from the canal is a good sign; early emigration of leucocytes is also a good sign; early emigration, active phagocytosis and dissolution of the diplococci, both within and without leucocytes, are very favorable signs. Proceeding in this way, I have been able to keep a small number of monkeys in a state of subinfection with the diplococcus for a period of several days or weeks. The period of successive inoculation was determined by the puncture and by the physical condition of the monkey. A state of resistance tended to develop which necessitated the employment of increasing amounts of cultures to produce visible effects; and in the end the run-down animal succumbed to the dose administered. In these animals the exudates were thicker and firmer and covered the base and the convex surface of the brain; and the ventricles were sometimes widely dilated and contained turbid exudates. The first of these experiments was made with a South American species of which the next protocol gives an account.

Monkey No. 27. South American; Genus Cebus.—October 7, 1905, 11 A. M. Injected suspension of one agar culture "Whitaker," three days old. No symptoms developed. October 9, 11 A. M., l. p.: free flow of turbid fluid, of which 1 c.c. was collected. Injected one "Smith" culture eighteen hours old. 1 P. M., animal crouched in corner; head down and resting in hands; indisposed to move. 5:30 P. M., condition about the same; l. p.: a small quantity of fluid containing leucocytes, but no diplococci obtained. Culture negative. October 12, 3 P. M., l. p. yielded a small amount of turbid fluid containing vacuolated leucocytes and mononuclear cells; no diplococci. One half agar slant culture of "Smith" injected. 9:30 P. M., animal depressed. October 13, animal on perch. October 16, appeared to be well. Suspension of two old "Smith" cultures injected. October 17, no marked effects. L. p. gave a few drops of faintly turbid fluid showing leucocytes with intact nuclei, of which some contain degenerated diplococci. October 18, l. p.: faintly turbid fluid flowed freely from the needle. C. s.: leucocytes and

mononuclear cells; no cocci. A number of vacuolated (or fatty) large mononuclear cells present. Injected one twenty-four hour culture "Smith." No symptoms developed. October 20, 3 P. M., l. p. gave small amount of turbid fluid showing leucocytes but no cocci. Culture negative. Injected one culture "Smith" twenty-four hours old. No symptoms. October 23, l. p.: 0.5 c.c. turbid fluid obtained containing leucocytes and a few red corpuscles. October 28, l. p. gave clear fluid containing mononuclear cells. Injected two "Smith" cultures. October 30, l. p.: a drop of turbid fluid obtained showing leucocytes with fragmented nuclei but no cocci. Injected two "Smith" cultures. October 31, l. p. yielded a few drops of turbid fluid showing many leucocytes, and large mononuclear cells; no definite cocci; culture negative. During this treatment the monkey lost in weight. No further injection was given until November 15, at 12:30 P. M., at which time a heavy suspension of a "Smith" culture, eighteen hours old (representing four agar slant surfaces) was injected. Symptoms rapidly developed; at 2:30, animal sick; l. p. gave with difficulty a few drops of blood-tinted fluid containing many agglutinated diplococci and a small number of leucocytes, of which some had included diplococci. 4 P. M., l. p. gave, with difficulty, little fluid in which diplococci were numerous. October 16, 9 A. M., monkey died. Autopsy. No excess of fluid in the meninges of the spinal cord, and no exudate was visible. Turbid fluid, in excess of the normal, infiltrated the meninges from the medulla to the optic commissure. The anterior and lateral pial surfaces of the hemispheres showed smaller and larger ecchymoses. The ventricles contained small quantities of turbid fluid; they were not dilated. Cultures from different parts of the brain and cord remained sterile. The cover-glass preparations from different levels of the spinal cord, the base and convexity of the brain, choroid plexus and ventricles differed from one another only in minor details. Several striking conditions were shown by them to exist in the membranes. In the first place, no, or almost no, normal cocci remained, but only fragments of swollen and degenerated cocci which were entirely or nearly so within cells. The cells consisted of polymorphonuclear leucocytes which predominated in number and contained the far greater number of cocci; and of much larger cells of wholly different aspect. The latter were several times as large as the leucocytes, and occasional examples were of colossal size (equal to 20 to 40 leucocytes). The nucleus was single, as a rule, and oval or crescentic in form, and vesicular. The protoplasm was pale, and the membrane at the periphery stained deeply. The smaller of these cells contained at times, degenerated cocci; and all may have ingested a variable number of ordinary leucocytes, or mononuclear cells. They were most numerous in the smears prepared from the exudate of the meninges of the medulla. The sections of the central nervous system present quite a different appearance from the other cases described. Although the exudate is thicker than in the previous instances, the chief novelty lies in the great proliferation of the fixed cells of the pia-arachnoid. The multiplication has taken place chiefly along the surface of the brain and at the outer line of the membrane. The new cells are relatively large and vesicular, and their occurrence in double rows gives at times a somewhat glandular appearance to the membrane. Polymorphonuclear leucocytes are numerous and distributed irregularly among the other cells. The degree

of encephalitis is far less than in many of the experiments. The main arterial branches of the brain show a more or less marked infiltration of the intima with leucocytes, and leucocytic invasion of the sub-intimal and inner-muscular layers of the vessels.

This experiment brings out the remarkable capacity which can be developed in the monkey to dispose of cultures of the diplococcus injected into the spinal canal. It is shown by the experiments that the actively bactericidal cells and secretions of the canal are capable of destroying, in a few hours, prodigious numbers of the diplococcus, and that agglutination of the diplococcus can also take place in the canal. Finally, it is shown that the monkey can be killed by an overdose of culture which causes death by poisoning and not by infecting the animals directly; for the lethal effect occurred in this case, although the diplococci were themselves quickly killed. Increased power, therefore, to destroy the diplococcus is not associated necessarily with an equally increased power to resist the toxic effects of the intracellular poison. This fact must have an important bearing upon a specific therapy of diplococcus meningitis.

The experiment is important in respect to the histological alterations present in the meninges. In no previous experiment did the exudate contain so large a number of cells which differed from leucocytes. The large cells described here occur regularly in cases of human meningitis and have been frequently observed. Their occurrence in numbers in this monkey serves to knit even more closely the experimental and the human diseases. Flexner and Barker,[11] and Councilman, Mallory and Wright[12] describe and figure these large cells among the leucocytic infiltration, and the latter investigators have traced their origin to the cells of the connective tissue and the lymph spaces of the pia-arachnoid. The histological conditions present in the membranes are comparable with those observed in cases of meningitis of longer duration in man.

Monkey No. 32. Macacus rhesus. April 19, 1906, at 12 M., given one agar culture "548" in the third generation. 3 P. M., l. p., no fluid obtained. April 20, 12 M., l. p., small quantity of clear fluid containing few leucocytes and free cocci. 6 P. M., animal depressed. April 21, active. April 22, 12 M., injected

[11] A contribution to our knowledge of Epidemic Cerebro-Spinal Meningitis. *Amer. Jour. of Med. Sciences* 1894, CVII, 155, 259.

[12] *Op. cit.*, p. 102.

one culture; no apparent effect. April 23, 4 P. M., l. p., very little fluid obtained. April 25, 12 M., one agar culture "Gratz." Fluid obtained before injection which produced no visible effect. April 26, one agar culture "Gratz." No symptoms. May 1, 3 P. M., l. p., yielded slow flow of faintly turbid fluid showing leucocytes and larger cells but no cocci. Injected turbid suspension (equal to four agar cultures). 6 P. M., animal sick. Died during the night, May 2. Autopsy: The spinal membranes contained an excess of fluid. On opening the dura mater a gelatinous exudate was seen to occupy the meshes of the pia. A similar exudate covered the base of the brain and surrounded the foramen of Magendie. The convex surface of the brain was greatly altered by a moderately firm and adherent exudate of white color which occupied the pia-arachnoid. It covered the surface of the convolutions and filled the sulci, thus giving a remarkably smooth appearance to the brain (Plate IV, Fig. 6). The ventricles were considerably dilated and contained an excess of turbid fluid. Smear preparations showed the chief accumulation of leucocytes to be at the base of the brain and the lower portion of the spinal cord. The lateral surfaces of the brain showed fewer and more were present in the choroid plexus than in the fluid of the ventricles. Diplococci were present everywhere; they were intracellular and often degenerated. The number varied: they were abundant at the base and less numerous at the convexity of the brain. The mucous membranes of the nose showed, among the columnar epithelium, a number of fragmented leucocytes carrying diplococci indistinguishable from those of the brain (Plate V, Fig. 7). Sections of the spinal cord and brain bear out the findings described. The exudate occurs throughout the membranes covering these structures. It is thicker over the posterior than over the anterior surface of the cord, and while it is abundant over the convolutions, it is especially thick in the sulci. Little or no fibrin occurs. The exudate consists chiefly of polymorphonuclear leucocytes among which there are a large number of moderately large mononuclear cells. The brain substance is remarkably free from infiltration, while the lumbar cord shows invasion, about its posterior surface, to a considerable depth. The dura of the cord at certain levels shows leucocytic infiltration. Diplococci which are very numerous in the exudate, are almost entirely within leucocytes. They have been carried by these cells into the substance of the spinal cord.

A striking feature of the sections is derived from the width of the ventricles. As a rule, these appear as slits in the sections; in this case they are wide cavities. Usually, the ependymal epithelium is regular and relatively high; in this case, it is often depressed or flattened, and a considerable flattening of the choroid plexus, toward the wall of the ventricle, is noticeable. A considerable degree of sub-epithelial cellular proliferation has taken place in the walls of the lateral and fourth ventricles. Leucocytes are moderately abundant in the ventricles.

The dura mater was removed with its attachments to the ethmoid bone, and longitudinal sections prepared. The leucocytes surround the olfactory bulbs in dense masses, and they push along clefts and spaces in the dense membrane, existing now as elongated columns and now in circumscribed, abscess-like masses.

This experiment is valuable in showing that a condition of subacute infection of the meninges can be procured in the monkey by

the use of extraordinary means, and when the condition is present and the thick exudate incidentally covers and occludes the foramen of Magendie, a degree of internal hydrocephalus develops. The experiment enforces a point already made. The histological study of the dura mater at the anterior extremity shows that leucocytes pass into its substance for a considerable distance beyond the proper confines of the cranial cavity. The cells could, indeed, easily carry diplococci with them, in which case an explanation for the occurrence of leucocytes carrying diplococci in the nasal mucosa might be readily found.

The series of experiments described suffices to establish the varieties of effects which are produced upon the central nervous system of lower monkeys by means of intra-spinal injections of cultures of *Diplococcus intracellularis*. Among the histological appearances mentioned, there was one monkey, No. 17, which deserves more particular attention. I described, in this case, a condition of acute endarteritis of the type frequently found in cases of human meningitis. It would appear that this pathological condition occurs in many forms of meningitis as is shown by the investigations of Hektoen,[13] Councilman, Mallory and Wright,[14] and others. The latter authors state, indeed, that it is less commonly found in the epidemic (*Diplococcus intracellularis*) form of meningitis than in other forms (pneumococcus, etc.) of the disease. They figure a slight degree of the condition in a goat in which they produced experimental meningitis by injecting the diplococcus. I have encountered in still another monkey inoculated with the diplococcus very extensive acute endarteritis. The case will be described in full in my article on a serum therapy for experimental meningitis. A spider monkey (*Atelas ater*) was inoculated on December 2, 1905, at 12 o'clock noon, with a slant agar culture of the diplococcus. At 3 P. M. two cubic centimeters of anti-goat (diplococcus) serum were injected into the spinal canal. Death occurred December 3, 11 A. M. The lesion mentioned affects arteries of all sizes in the brain. At the base, over the medulla, rarely does

[13] The Vascular Changes of Tuberculous Lepto-meningitis, especially the Tuberculous Endarteritis, *Jour. Exp. Med.*, 1896, I, 112.

[14] *Op. cit.*, p. 77.

an artery escape. The arteries of the convexity are affected less uniformly, and those of the spinal cord least of all. The systemic arteries do not show the lesion. The subendothelial infiltrations of mononuclear and polynuclear cells may or may not occlude the vessel entirely (Plate V, Fig. 8). Both large and small vessels may be partially or wholly filled up with cells which, as a rule, push the endothelium before them. The endothelium is preserved unless lost from mechanical causes in making the sections. An appearance has been obtained in some vessels of a mushroom-like overlying of the endothelium by the new cells which have accumulated in the adjacent part of the vessel. The muscular coat is usually free from infiltration, as are the veins. This monkey showed an unusual degree of hæmorrhage into the meninges, and definite cortical abscesses, of small size, extending into the brain from the cortical membranes, or developing about the intra-cortical blood-vessels. The number of diplococci in the inflammatory exudate was not large.

The pathological effects and appearances which have been described are not, probably, specific. The experiments are, therefore, valuable, not because they show that *Diplococcus intracellularis* alone of the pyogenic micrococci can produce acute meningitis in monkeys, but because they prove that the diplococcus is capable of setting up in these animals lesions of meningitis which bear a close resemblance to the lesions which the same micro-organism causes naturally in human subjects. The finer the details of correspondence are between the natural and experimental disease, the more valuable do the experimental results become, and the more convincing are they that *Diplococcus intracellularis* is really the cause of epidemic meningitis in man. I have not undertaken to study all the common pyogenic micrococci in the manner of this study of the diplococcus, but I have made, for obvious reasons, a single experiment with *Micrococcus catarrhalis.* The culture employed was kindly given me by Dr. Elser. It fulfilled the usual biological tests for that micro-organism.

Monkey No. 28. South American; Genus Cebus.—October 12, 1905, at 3 P. M., inoculated three agar slant cultures of *M. catarrhalis.* Clear fluid flowed from the needle before the injection. 9 A. M., animal sick; sat on

bottom of the cage. October 13, 9 A. M., condition unchanged. 3 P. M., very sick; head rested on hands. L. p., 9 A. M., yielded a very small quantity of fluid by using syringe. C. s., many leucocytes containing intracellular cocci in moderate numbers; a few cocci extra-cellular. The cocci appeared larger than the diplococcus; some stained feebly. October 16, animal had lost flesh but was recovering. Sat on perch. L. p., 3 P. M., with the syringe a few drops of faintly turbid fluid obtained. It showed small numbers of leucocytes with fragmented nuclei, but no cocci. This animal never fully recovered its flesh, and the hair entirely disappeared from the long tail. It was found dead on February 12, 1906. The autopsy showed the brain and cord to have returned, apparently, to the normal condition. Cover-slips were free of leucocytes and cocci; cultures remained sterile. An adequate cause of death was not found. Nearly all of this species of monkey contain adult filaria in the peritoneal cavity, and filarial embryos in the blood.

. . . . . .

By means of the experiments recorded in this paper, it has been shown that the lower monkeys can be infected without great difficulty with *Diplococcus intracellularis* and made to reproduce the pathologic conditions present in man in cerebro-spinal meningitis. The experiments establish that the diplococci, when introduced in a low level of the spinal canal, distribute themselves in a few hours through the meninges and excite an acute inflammation, the exudate of which accumulates chiefly in the lower spinal meninges and the meninges of the base of the brain. The uniformity with which the chief exudate is found at the base of the brain and the rarity of its appearance in equally great amounts over the convexity is a fact of importance in the dynamics of the cerebro-spinal fluid since there is relation between the quantity of exudate and the accumulation of the diplococcus. This distribution of the exudate led me to doubt the validity of the reasoning which would ascribe this localization of the inflammation in man to the entrance into the meninges of the infective agent directly through the nasal membrane. This tendency to localization at the base of the brain in monkeys is especially interesting in view of the fact that it is usually only a short time before death that they lie down upon the side of the body. In comparing the experimental lesions in monkeys with the naturally developed lesions in man, note should be taken of the occurrence under both conditions of encephalitis and abscesses, of hæmorrhages, of proliferation of large cells of connective tissue and tissue spaces, of acute endarteritis, of inflammation of the dorsal

root ganglia, of internal hydrocephalus, of relatively small amount of fibrin in the exudates, of fibrinous and other thrombi, and of phagocytosis of diplococci and somatic cells.

The inflammation of the meninges extends in monkeys into the membranes covering the olfactory lobes and along the dura mater into the ethmoid plate and nasal mucosa. The nasal mucous membrane is found, in many instances, to be inflamed and beset with hæmorrhages. Smear preparations from the nasal mucous membrane, from the higher levels especially, have shown me polymorphonuclear leucocytes, often in numbers, carrying diplococci which presented the form, size, staining qualities and degenerations of the diplococci occurring in the same cases in the cerebral and spinal meninges. Thus far *Diplococcus intracellularis* has not been cultivated from the nose of the infected monkeys. I have, however, secured other Gram-negative diplococci with which, it may be stated, the intracellularis could not be confounded.

Although the pathological effects produced in monkeys are comparable with those occurring in the natural disease in man, there is no real correspondence in the relative degree of susceptibility of the two species. The quantity of an active culture required to cause marked symptoms or to bring about death from meningitis in monkeys, is prodigious if compared with the number of diplococci which probably suffice to produce infection in human beings. Moreover, the amount of multiplication of the diplococcus in monkeys, excepting possibly in the focal abscesses, is under the most favorable conditions, small; and I am not disinclined to believe that in many of the experiments no multiplication whatever took place.

The experiments, the details of which have been set down here, besides being an argument for the causative action of *Diplococcus intracellularis* in epidemic meningitis, form, also, the basis of an attempt to influence the progress and termination of the experimental infection in monkeys through the employment of anti-sera prepared from the diplococcus. The results of the experiments with the anti-sera are given in a separate article.

I wish to acknowledge the valuable aid rendered by my former assistant, Dr. H. S. Houghton, in the course of these experiments.

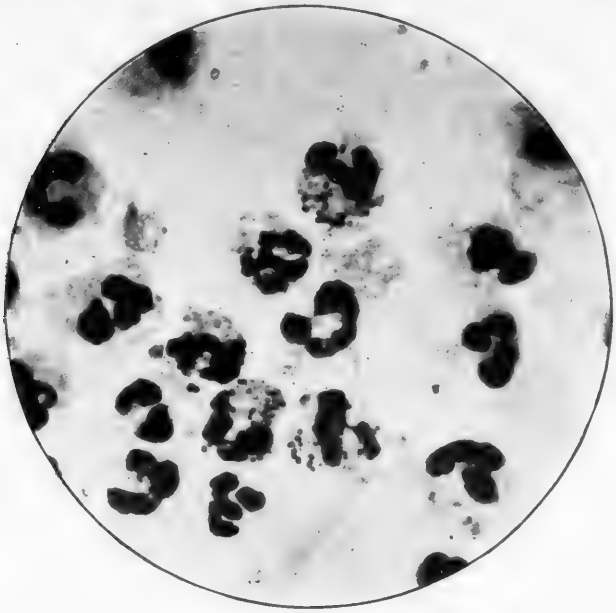

FIG. 1.

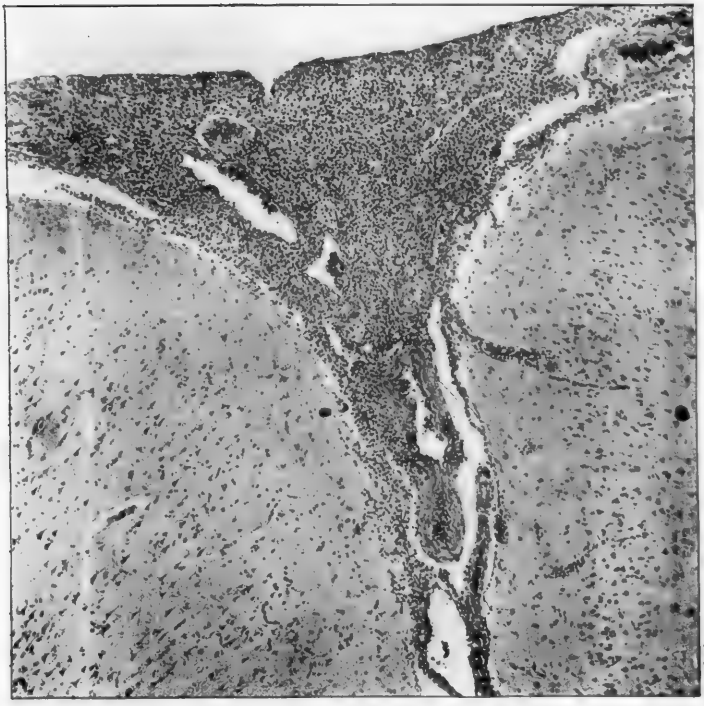

FIG. 2.

M40U

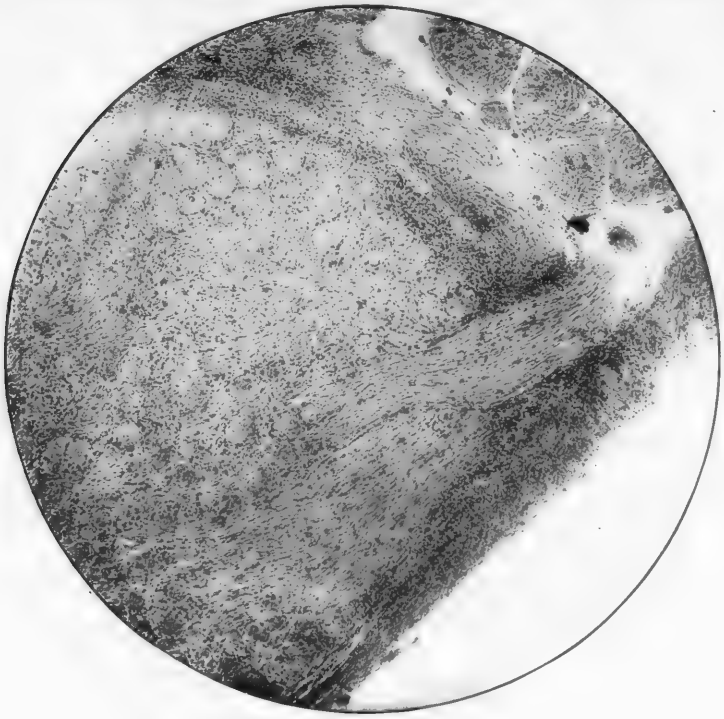

FIG. 3.

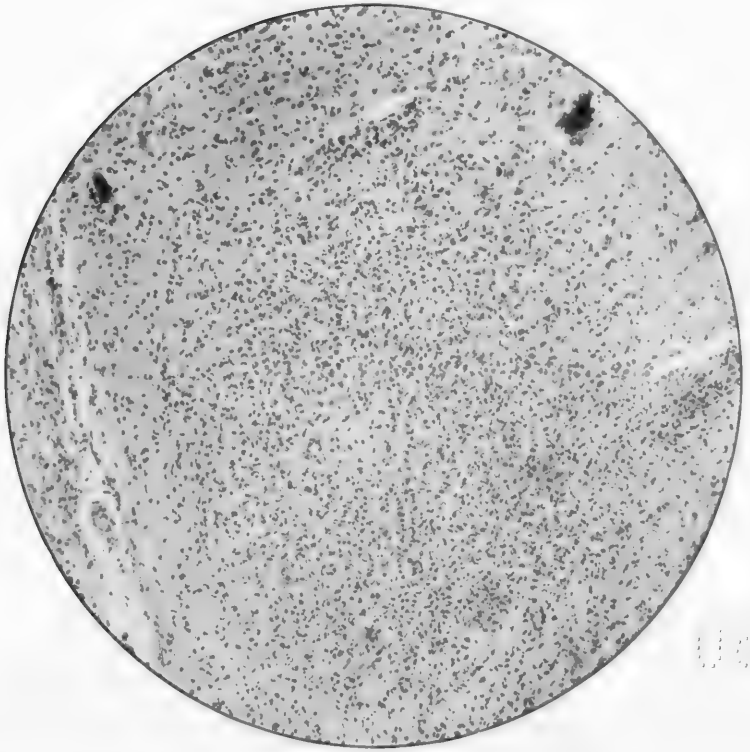

FIG. 4.

Uofm

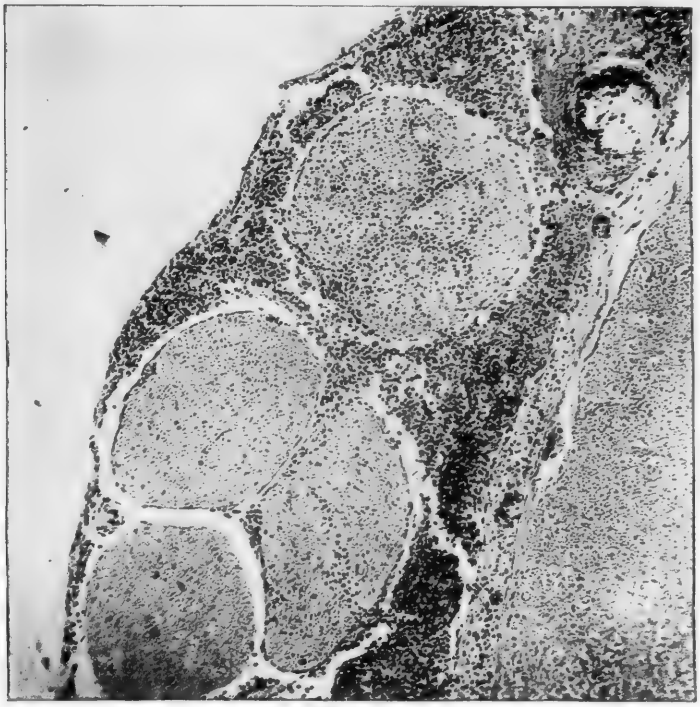

FIG. 5.

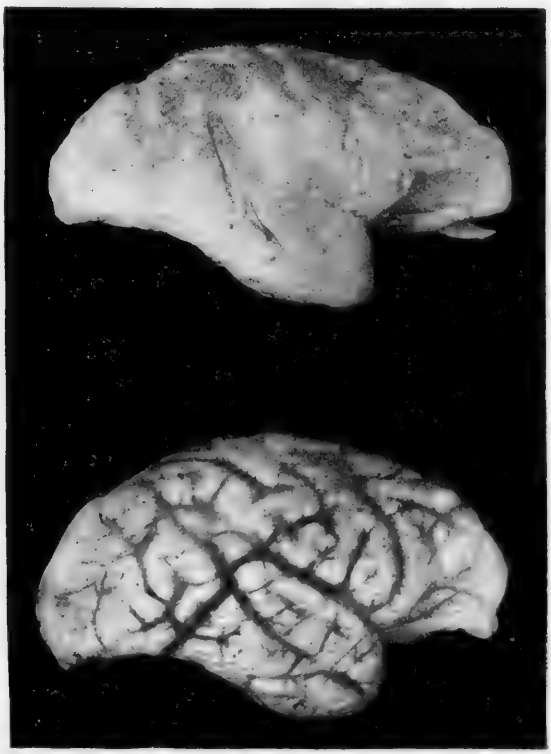

FIG. 6.

FIG. 7.

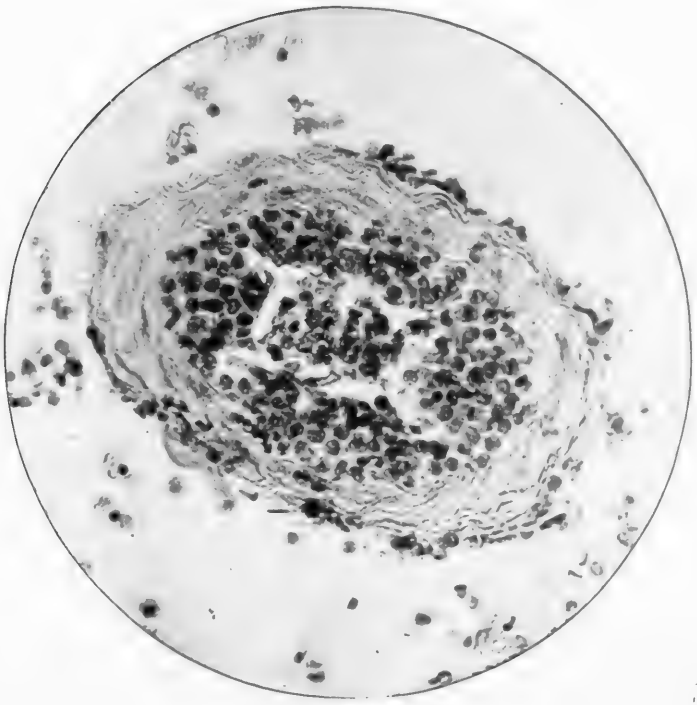

FIG. 8.

DESCRIPTION OF PHOTOGRAPHS.

Plate II.

Fig. 1. Smear preparation from contents of brain abscess, monkey No. 1. Typical intracellular diplococci. Methylene-blue staining. $\frac{1}{12}$ in. objective.

Fig. 2. Low magnification to show the relation of the exudation in the pia-arachnoid of the convex surface of the brain and the sulci, and the extension of the exudation along the cortical vessels into the brain.

Plate III.

Fig. 3. Spinal ganglion showing leucocytic invasion about and between the nerve-cells of the ganglion.

Fig. 4. Low magnification to show the degree of acute encephalitis, and the involvement of intracortical blood-vessels in the infectious processes.

Plate IV.

Fig. 5. Low magnification to show the exudation about and within the spinal nerves.

Fig. 6. Brain, natural size, from a case (Monkey No. 32) of subacute meningitis compared with the brain from a case of very acute experimental meningitis in which the congestion of the pial vessels is very marked.

Plate V.

Fig. 7. Smear preparation from inflamed nasal mucosa showing a leucocyte, partly broken, enclosing diplococci, lying among columnar ciliated epithelial cells.

Fig. 8. High magnification of an artery of the base of the brain showing the lesion of acute endarteritis.

I am indebted to the skill and kindness of Dr. Edward Leaming for the photographs illustrating this paper.

CONCERNING A SERUM-THERAPY FOR EXPERIMENTAL INFECTION WITH DIPLOCOCCUS INTRACELLULARIS.

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The high mortality of epidemic meningitis and the deplorable deformities caused by it demand that incessant effort be made to discover therapeutic measures which may mitigate the consequences of the disease. The epidemic through which the city of New York has recently passed, and the almost co-incident Silesian epidemic, have been scientifically fruitful in establishing more firmly the belief in the spread of the disease through immediate or mediate contact with the sick, and in tracing the common occurrence of *Diplococcus intracellularis* in the nasal and pharyngeal secretions of the sick, and the exceptional occurrence of the micro-organism in these secretions in the well who have been in contact with the sick. This mode of spread of the disease through directly and indirectly infected persons must come to exercise an important influence on the hygienic measures which will be enforced hereafter to limit the dissemination of the disease.[1]

[1] Goodwin and Sholly: The frequent occurrence of meningococci in the nasal cavities of meningitis patients and of those in direct contact with them. *Journal of Infectious Diseases,* 1906, Supplement No. 2, p. 21.

Flatten: Die übertragbarre Genickstarre im Regierungsbezirk Oppeln, im Jahre 1905 und ihre Bekämpfung. *Klinisches Jahrbuch,* 1906, xv, 211.

Schneider: Idem im Regierungsbezirk Breslau, *ibid.,* p. 300.

Rieger: Idem im Kreise Brieg, *ibid.,* p. 321.

Schmidt: Idem im Regierungsbezirk Leignitz, *ibid.,* p. 341.

Flügge: Die im hygienischen Institut der königl. Universität Breslau während der Genickstarre-epidemie im Jahre 1905 ausgefuhrten Untersuchungen, *ibid.,* p. 353.

v. Lingelsheim: Die bacteriolog. Arbeiten der kgl. hyg. Station zu Beuthen, etc., *ibid.,* p. 373.

Göppert: Zur Kenntnis der Meningitis cerebro-spinalis-Epidemica mit besonderer Berücksichtigung des Kindersalters, *ibid.,* p. 313.

A less certain advance has been made in the therapeutics of epidemic meningitis. The one therapeutic measure growing out of the study of the epidemics in America and Germany which offers any hope is an antiserum for the diplococcus. It is true that the experience of the past is not favorable to the hope of achieving remarkable success by the employment of antibacterial immune sera. All indications point to the pathological effects of the diplococcus as being caused by endotoxic constituents; and thus far, according to many investigators, these endotoxins have failed to yield, by methods of immunization, active antisera which have proved valuable in the treatment of infectious diseases. Opinion is, however, considerably divided on this subject;[2] and in the absence of more certain methods of reaching the desired goal tests of antisera for the diplococcus are certainly justified. These tests can in the preliminary stages be carried out on certain animals, since the course of infection in them with the diplococcus is now fairly well understood.[3]

The main question which would seem to be involved in the search for an active antiserum against meningitis is whether the quantity of antibody which can be produced will suffice to neutralize such a quantity of the poison of the diplococcus as to influence the result of the infection. In fact, the problem may not be so simple, or, indeed, so hopeless, as this proposition indicates. It is, of course, important that neutralization of the poison should if possible be secured, but the effect of the restraint of growth and multiplication of the diplococcus may, at some periods of the disease, be of greater significance than the neutralization of free endotoxin. Fortunately, many agents, some of them quite indifferent, are able to affect the power of multiplication in the body of the diplococcus. It has been shown, indeed, that serum in the fresh state and after

Meyer: Bericht über rhinolog. Beobachtung bei der Genickstarre-epidemie, 1905, *ibid.*, p. 427.

Westenhoeffer: Pathologisch-anatomische Ergebnisse der oberschlesischen Genickstarre-epidemie von 1905, *ibid.*, p. 447.

Jehle: Entstehung der Genickstarre-epidemie, Wien. klin. Woch., 1905, xix, 25.

[2] Besredka, *Annales l'Institut Pasteur*, 1906, xx, 4.

[3] Attention is directed to the two previous papers of this series published in this number of the Journal.

heating to 60° C., preserves the power to destroy in test-tubes large numbers of the diplococcus, and sterile fluid inflammatory exudates possess this power in even greater degree. An antiserum, therefore, even though it contain relatively small amounts of antibodies, as indicated by neutralization experiments, may be effective beyond this calculated value by restraining the multiplication of the diplococci, possibly by reducing outright their number, and by supporting the power of resistance normally at the disposal of the body.

The conditions are made theoretically less discouraging, perhaps, because the main pathological lesions are limited to the cavity of the cerebro-spinal axis. They can, therefore, be brought directly under the influence of the antisera by injecting the latter into the spinal canal. A large advantage is gained by this circumstance. It is, on the other hand, discouraging to reflect that in monkeys infected with the diplococcus, severe cortical lesions already exist at the end of ten or twelve hours. The question arises whether these deeper lesions tend to appear as early in the human infections. In respect to this question it should be stated that observation is against the occurrence of any such development of the diplococcus in the early stages of the human disease as is represented by the prodigious number of diplococci required to be injected into monkeys to produce the rapidly lethal effect with which the cortical lesions are associated. It is worthy of note that the more slowly developed lesions in the monkey remain more superficial, agreeing in this respect with the more common lesions present in fatal cases of the human infection. Hence, some encouragement may be taken from the power of the antiserum to influence favorably the course of meningitis in the monkey, although it has been injected as late as six hours after the inoculation.

If we are at all permitted to apply test-tube experiments to what may happen in the body, it would not be remarkable if the normal serum of animals, and perhaps of human beings, proved to be beneficial to a degree when brought into direct relation with the focus of development of the diplococcus. At first sight, judging from test-tube experiments, it would appear as if the exudate, called out by the inflammation, should suffice to destroy the diplococci; this manifestly does not happen in many cases. Indeed, it

is found that incubation outside the body will even increase the number of diplococci in the inflammatory fluid withdrawn from the spinal canal. It is safe to assume, therefore, that the exudate withdrawn has been exhausted of its power to destroy the diplococcus. It is quite possible that the introduction of fresh serum, of the same species of animal, may be helpful by bringing quickly into contact with the diplococci a quantity of actively destructive serum. The results of some of my experiments show that normal serum reduces appreciably the toxic effect of given doses of the diplococcus.

In experiments upon the monkey there is a definite low limit, beyond which it is not safe to go, for injection of fluids into the spinal canal. The species which I studied contain a small amount only of free spinal fluid. If one attempts to inject several cubic centimeters of fluid, symptoms of pressure may develop. In this respect the monkey is far less satisfactory to treat by intraspinal injections than are human beings.

I am far from having any conviction that cerebro-spinal meningitis in man can be influenced favorably by injections of immune sera into the spinal canal, or elsewhere in the body. The experiments to be described merely show that guinea-pigs and monkeys, in which the conditions of infection can be controlled, can be saved from the otherwise fatal effects of the diplococcus by the use of antisera, and to a less extent by the use of normal sera and other fluids. A preliminary note on this subject has already been made.[4] The protocols show that the experiments on immunity were begun during the spring of 1905. While the work was in progress two papers on the same subject appeared in Germany.[5] The use of monkeys for testing the antisera by direct injections into the infected and inflamed cerebro-spinal canal has not been made by the other investigators whose experimental studies were confined to guinea-pigs. Jochmann injected an antiserum prepared in the horse into the spinal canal of several human subjects of epidemic meningitis. The number of cases was too few to permit any conclusion of the value of the injections; but they showed that the injection of

[4] *Jour. of Amer. Med. Assoc.*, 1906, xlvii, 560.

[5] Kolle and Wassermann, *Deutsch. Med. Wochenschrift*, 1906, xxxii, 16. Jochmann, *ibid.*, p. 20.

horse's serum into the inflamed canal is not attended with special danger.

My first experiments on guinea-pigs were made with goat's sera. A female goat had been injected twice with cultures from several sources (12) of the diplococcus within a period of two weeks. The injections were made subcutaneously and gave rise to tumefaction which soon disappeared. After the second injection the goat aborted. The first bleeding was made two weeks after the second injection. As the table shows the serum at this time had little or no immunizing power. The experiment was designed to test the effect of an injection (1) previous to the injection of the diplococci, and (2) after the inoculation of the diplococci. The serum in the first instance was injected at 5 P. M. the day before, and in the second instance two hours after the inoculation with the diplococcus. All the injections, except bouillon in one pig, were intraperitoneal. The emulsion of the diplococcus was injected at 11 A. M., November 29, 1905.

Series No.	Weight in Grams	Protective Substance Injected.	Result.
144	189	"Immune" serum 0.03 c.c. 5 P.M. Nov. 28	Died 9 A.M. Nov. 30.
145	189	" " 0.04 c.c. " " "	Died 8 A.M. Dec. 2.
146	182	" " 0.05 c.c. " " "	Survived.
147	197	"Normal" " 0.05 c.c. " " "	Survived. Lost weight.
148	197	" " 0.10 c.c. " " "	" " "
149	220	Bouillon intraper. 1.0 c.c. " " "	" Weight 12/11 182 grams.
150	199	" subcut. 1.0 c.c. " " "	Died morning, Dec. 3.
151	190	"Immune" serum 1.0 c.c. 1 P.M. Nov. 29	Survived. Lost weight.
152	185	" " 2.0 c.c. " " "	" " "
153	192	"Normal" " 1.0 c.c. " " "	Died 5 P.M. Nov. 30.
154	190	" " 2.0 c.c. " " "	Died in night, Nov. 29.
155	190	Nothing: control	Died morning, Dec. 1.
156	210	Idem.	Died in night, Nov. 29.

The preceding experiment is of interest in showing the irregular action of the serum injections, and especially, as bringing out the fact that such an indifferent substance as bouillon can, if it is injected in advance of the diplococcus, impart power of successful resistance to the guinea-pig. It developed subsequently that the bouillon need not be injected into the peritoneal cavity to achieve this effect; one cubic centimeter injected subcutaneously in pigs of 225 to 250 gram weight, the day before inoculation, frequently

saves the animals. Using the goat serum of low protective value, the fact was determined that the protecting power of the serum has a definite limit (about 0.05 c.c. for pigs of 250 grams weight) under the most favorable conditions, namely, intraperitoneal injection the day before the infection. By using larger quantities (up to 1.0 c.c.) of serum simultaneously with the injection of the diplococcus the pigs can also be saved; subcutaneous injections of the serum (1.0 c.c.) in advance are effective. The smallest number of successful results is obtained in pigs in which the serum injections follow, after one or more hours, the infection. Several experiments were carried out in order to determine the fate of the diplococcus in the peritoneal cavity in the "protected" and "unprotected" animals. The plan was to withdraw fluid, after a suitable interval, by means of capillary tubes. The results were not wholly uniform, but in the main showed more rapid disappearance of the

Guinea-pigs weighing 230 to 260 grams, received 0.1 c.c. "immune" goat's serum intraperitoneally and subcutaneously, 1.0 c.c. bouillon intraperitoneally and subcutaneously, followed the next day by an emulsion of the diplococcus injected into the peritoneal cavity.

Series No.	How Injected.	Examination During Life.	Autopsy Findings in Peritoneum.
119	Goat's serum intraperitoneal; emulsion cocci at 11:30 A.M.	4 P.M. Many leucocytes containing a few diplococci; no extracellular diplococci.	Survived.
120	Goat's serum subcutaneous; emulsion cocci at 11:30 A.M.	4 P.M. Enormous number of diplococci; very few leucocytes overlaid with diplococci.	Died during the night. 2 c.c. fluid exudate; more pus present than usual; many leucocytes including diplococci.
121	Bouillon intraperitoneal; emulsion cocci at 11:30 A.M.	4 P.M. Diplococci more numerous than in 120; no leucocytes.	Died during the night. 3 c.c. fluid exudate; no leucocytes; many diplococci.
122	Bouillon subcutaneous; emulsion cocci at 11:30 A.M.	4 P.M. Many leucocytes; some intracellular diplococci; very few extracellular ones.	Survived.
125	Control; emulsion cocci at 11:30 A.M.	4 P.M. Enormous number of diplococci; almost no leucocytes.	Died during the night. Usual p. m. appearances; very large number of diplococci free; almost no leucocytes. The omentum contains some leucocytes including cocci.

diplococcus from the peritoneum of the treated as compared with the untreated pigs. Exudation of cells was more abundant in the former animals.

The injections of the goat were carried on subsequently, during and after lactation, until the end of February, 1906. Blood was withdrawn from time to time and the serum tested for its protective and therapeutic value. The results were never uniform, but the general indication was that the protective properties were increased measurably. The animal fell ill on March 15, 1906, and was bled to death. The serum obtained from this bleeding was used for many subsequent experiments. It was found by simultaneous injection to protect small pigs (190 to 200 grams) against a twelve hour fatal dose of the diplococcus in quantities varying from 0.5 to 0.01 c.c., but not regularly. On the whole, as the next experiment given in detail will tend to show, the serum had acquired protective and therapeutic properties.

An emulsion of the diplococcus was prepared of which 0.5 c.c. caused death in the control pigs in about eighteen hours. Three series of pigs were tested; the first received the serum and emulsion simultaneously, the second the serum two hours, and the third the serum four hours after the emulsion. One tenth c.c. of serum was injected in each pig. The emulsion was given intraperitoneally.

Series No.	Serum Injected.	Result.
102	Intraperitoneal; simultaneously.	Survived.
103	Subcutaneous; "	Died in 4 hours.
105	Subcutaneous; after 2 hours.	" 16 "
106	Intraperitoneal; " 2 "	Survived.
107	Subcutaneous; " 4 "	Died in 56 hours.
108	Intraperitoneal; " 4 "	Survived.

I shall supplement this table by another in which the goat serum is compared with an immune serum made in monkeys (vide infra). This experiment has, in this place, a two-fold value in showing that certain of the series of tests proceed in a regular manner, and the two immune sera have about equal protective value for guinea-pigs. The emulsions of the diplococcus killed six control pigs in from nine to fifteen hours. The injections were simultaneous.

The next table given shows that the sera of the goat and the monkey were both capable of protecting guinea-pigs from fatal doses of the diplococcus by simultaneous injection, and the previous

Series No.	Weight in Grams.	Serum Injected.	Result.
106	190	0.1 c.c. goat, interperitoneal.	Survived.
107	188	Idem.	"
108	180	0.1 c.c. monkey, intraperitoneal.	"
109	185	Idem.	"
110	185	0.1 c.c. goat, subcutaneous.	"
112	175	Idem.	Died in 12 hours.
114	195	0.1 c.c. monkey, subcutaneous.	Survived.
117	178	Idem.	"
175	210	0.1 c.c. goat serum, intraperitoneal.	"
178	205	0.2 c.c. " " "	"
181	170	0.5 c.c. " " "	"
188	185	1.0 c.c. " " "	"
182	205	0.1 c.c. monkey serum, intraperitoneal.	"
183	182	0.2 c.c. " " "	"
186	175	0.5 c.c. " " "	"
187	172	1.0 c.c. " " "	Died in 30 hours.

one indicates that the infected pigs can with less uniformity be rescued at the expiration of two to four hours after inoculation. The striking results of the last table are, however, relative merely, since it is found that certain normal goat sera possess a power that it almost equal to that of the immune serum in protecting by simultaneous inoculation. On reweighing, four days later, the surviving pigs of the last tabulation a marked loss of weight was found to have taken place in those which received the sera subcutaneously and not in those receiving it into the peritoneum.

It would be extremely hazardous to express, on the basis of my experimental results, the relative value, as protective agents against the diplococcal infection in guinea-pigs, of normal and immune goat sera. I was restricted to the use of a female goat for the purpose of immunization, which was, unfortunately, during a part of the process, lactating. This fact could account for the low relative value of the serum. I did not, however, find the injection of normal goat serum following the inoculation of the diplococcus, so effective as the injection of the immune serum in this way. But here again, I was, I should state, limited in this study to three specimens of normal serum. On the whole, the immune serum saved more guinea-pigs than the normal serum. I am not, however, convinced that in the instances of preceding or simultaneous injection of the serum, in respect to the inoculation, any very great stress can be laid upon the inherent antitoxic, or even bactericidal power of the specific serum, since, in the former case, the exudate

caused to be poured in the peritoneal cavity has itself, in vitro, the power of suppressing large numbers of diplococci, and, in the latter, the sera possess this power only in less degree than the exudate. If, however, it can be shown that the immune sera are more effective as therapeutic agents than normal sera, and by simultaneous and preceding injections, also, then it will have to be conceded that they contain some useful elements absent from the normal serum or present in it in less amount.

In the course of these experiments the effort was made to produce an immune serum in the horse. For this purpose, recent cultures were injected by Dr. Jobling, first subcutaneously and later intravenously. The doses had to be carefully chosen on account of the high sensibility of the horse to the diplococcus. The subcutaneous injections produced local swellings which frequently softened and discharged externally. After a period, intravenous injections of autolysates were begun; but the alarming symptoms which followed almost immediately the injection of a few cubic centimeters of this fluid caused a return to the use of cultures and autolysates by the subcutaneous method. After about five months of intermittent injection the serum was collected and compared for protective value on small guinea-pigs. I employed as controls several samples of normal horse serum kindly supplied by Dr. Park of the Board of Health. I shall give a few typical experiments from which it would appear that on the whole the serum of the treated horse has greater protective value than the serum of normal horses.

Two strains of the diplococcus of moderate virulence were employed in the tests. The suspensions were made in the morning, control pigs being inoculated with them immediately. About four hours later, the rectal temperatures indicated the probably fatal doses. It was subsequently found that 0.1 c.c. of the suspensions caused death in less than twelve hours and represented, for susceptible pigs of about two hundred grams, two or three fatal doses. The inoculation of the pigs for the experiments on immunity was made in the afternoon of the same day. This procedure is rendered necessary by the rapid deterioration of the suspensions in salt solution and Ringer's fluid which sometimes takes place at

the refrigerator temperature. The deterioration is associated with loss of viability and not with striking morphological changes in the diplococci, from which fact I have been led to believe that multiplication for a period of the inoculated diplococci may be a necessary condition of the lethal dose.

Three control guinea-pigs were injected at 10 A. M. with 0.1, 0.2, and 0.3 c.c. of a suspension of "Mt. Sinai culture" of the diplococcus. The pig receiving the largest dose died at 4.30 P. M., at which time the temperatures of the other pigs were 34.6 and 34.8° C., respectively. A series of guinea-pigs which had been injected into the peritoneum at 4 o'clock the previous day with normal and immune horse serum, were now inoculated into the abdominal cavity with the suspension of the diplococcus. The two remaining control pigs died at 11.30 P. M. the same day. All the pigs weighed between 165 and 190 grams.

NORMAL HORSE SERUM.

Series Number	Quantity of Serum Injected 24 Hours Previously.	Quantity of Suspension Injected.	Result.
68	0.01 c.c.	0.2 c.c.	Died midnight.
69	0.02 "	" "	Died 3 A.M.
72	0.05 "	" "	Died midnight.
75	0.07 "	" "	Survived.
78	0.10 "	" "	Died midnight.
82	0.30 "	" "	Died 1 A.M.

IMMUNE HORSE SERUM.

47	0.01 c.c.	0.2 c.c.	Died 3 A.M.
50	0.02 "	" "	Survived.
58	0.05 "	" "	Died 9 A.M.
59	0.07 "	" "	Survived.
61	0.10 "	" "	Died 3 A.M.
66	0.30 "	" "	Survived.

This result, while it is not remarkably good, does, on the whole indicate that the immune serum possesses greater power of protection than the normal serum. The condition of the experiment was fairly severe, since the dose of suspension represented about five fatal doses; but what should be especially noted is the varying sensitiveness of the inoculated pigs owing to which the tests give quite irregular results.

The next tabulation, which would seem to bring out the same fact, gives the result in a series of simultaneous inoculations. The

mixtures of suspension and serum were permitted to stand at the warm room temperature for half an hour before injection.[6]

NORMAL SERUM NO. 1.

Series Number.	Serum Injected.	Suspension Injected.	Result.
51	0.01 c.c.	0.1 c.c.	Died after 8 hours.
54	0.02 "	0.2 "	" " " "
46	0.10 "	0.5 "	Survived.
	NORMAL SERUM NO. 2.		
49	0.01 c.c.	0.1 c c.	Survived.
43	0.02 "	0.2 "	"
38	0.10 "	0.5 "	Died after 8 hours.
	IMMUNE SERUM.		
41	0.01 c.c.	0.1 c.c.	Survived.
37	0 01 "	0.1 "	"
34	0.02 "	0.2 "	"
44	0.02 "	0.2 "	"
42	0.10 "	0.5 "	"
39	0.10 "	0.5 "	"

Two other kinds of antisera were prepared and tested upon guinea-pigs. The first was made by injecting large rabbits with the peritoneal exudate of guinea-pigs which succumbed to intraperitoneal inoculation with the diplococcus. The exudates were toluolized, freed from cells, the toluol was removed by evaporation at a low temperature, and injected into rabbits. The antisera obtained from the rabbit exercised a degree of protection which can be expressed as follows: simultaneous injections of the diplococcus and the antiserum into the peritoneal cavity tend to give protection; separate simultaneous injections, diplococcus into the peritoneum and serum under the skin, are effective in proportion to the quantity injected. Small doses of the serum (0.1 c.c.) do not protect, but they delay the lethal effect; larger doses (0.5 c.c.) prevent the lethal effect. Dosage of rabbit serum proved to be important; too little failed to protect, and too much (0.5 c.c. into the peritoneal cavity) prejudices the result by reason of its own toxicity. The pigs which received the culture and this dose of serum died soon after the controls. The second was an homologous serum

[6] It is my intention to make a later report, when the immunization shall have gone farther, upon the effects of the serum of the immunized horse as regards its power of protection for guinea-pigs and for monkeys.

yielded by large guinea-pigs which were injected at intervals for several months with cultures of the diplococcus, the peritoneal exudate from other guinea-pigs, and the autolysate, which proved not to have greater protective value than normal guinea-pig's serum.

If the result of these attempts to produce an antiserum for *Diplococcus intracellularis,* which should be effective in the experimental infection with the diplococcus in the guinea-pig are reviewed, they cannot be held to be particularly promising. Under the severe conditions of the experiments, the most that can be said is that various agents—bouillon, normal sera, immune sera—can at times affect favorably the course of the experimental infection; the lead in respect to this influence being taken rather by the immune sera. It is to be recalled that in small guinea-pigs the experimental infection is rapidly fatal; that the prostration of the pigs develops very quickly, and the animals are often moribund in six to eight hours after inoculation. To influence very favorably and systematically a pathological process which progresses as rapidly as this, would be, perhaps, an achievement. As the experiments show, this can be done, although not wholly in this degree, by appropriate dosage of certain antisera.

The next experiments to be reported relate to monkeys inoculated with the diplococcus and treated with anti-diplococcus serum made in the monkey. Two large monkeys (*Macacus nemestrinus*) were immunized, for the production of an homologous serum, by injecting them subcutaneously with exudates from the peritoneal cavity of guinea-pigs succumbing to diplococcus infection, and with emulsions of the diplococcus. The injections were made at intervals for a period of nine months, after which the animals were bled to death, and the sera tested. Before giving the protocols of these experiments, the chief facts of a much earlier experiment to influence the course of meningitis in a monkey by means of the antiserum of the goat should be given.

December 2, 1905, each of two spider monkeys (*Atales ater*) was inoculated with one agar slant culture of the diplococcus. Fluid flowed from the needles before injection. 3 P. M., control sick; mate to this perhaps not quite so sick. Into the spinal canal of the latter 2 c.c. of goat's immune serum were injected. The immediate effect was alarming: animal relaxed, heart's action tumultuous,

respiration sighing. The symptoms passed off in 10 to 15 minutes. L. p. before injection of serum showed in each many diplococci; no leucocytes. 9 P. M., control in stupor from which he could be aroused; easily handled. Responded to introduction of needle for l. p., no fluid obtained; point of needle carried a small amount of exudate which showed many leucocytes and a small number of intracellular cocci. Rectal temperature 40° C. Serum treated, less stupid than control; could be handled alone. No fluid obtained by l. p. The small amount of exudate on needle showed leucocytes some of which contained diplococci. Rectal temperature 36° C. December 3, 6 A. M., both still very sick; 8 A. M., serum treated animal very much depressed; 10 A. M., serum treated animal dead; control brighter. The latter animal finally recovered. Autopsy on serum treated monkey. The spinal canal showed hæmorrhagic imbibition of the membranes of the lower third of the cord. The pia-arachnoid of the cord was infiltrated with gelatinous-œdematous exudate. Small hæmorrhages beset the pia-arachnoid of the cortex, and the meninges of the brain were infiltrated with an exudate similar to that of the spinal cord. The ventricles contained turbid fluid in small amount. Microscopical examination of sections of the brain and cord show the exudate to be moderate in amount, and to be thicker over the convexity than over the base of the brain. The hæmorrhages in the membranes of the spinal cord are large, and of the brain small. The most striking lesion is an acute endarteritis which effects all sized branches of the arteries of the brain and cord. Very few diplococci can be found.

The symptoms in this animal and the lesions found at autopsy were taken to indicate that goat serum cannot be injected with impunity into the inflamed spinal canal of monkeys.

Preliminary to the tests of the antisera prepared in the two monkeys, the lethal dose of the diplococcus had to be established. A recently isolated culture (Mt. Sinai 596), which had proven virulent for guinea-pigs, was chosen. Two control monkeys were inoculated (1) with 0.5 c.c., (2) with 1.0 c.c. of a suspension of the culture. Brief histories follow:

Control No. 1. *Macacus rhesus.* June 27, 1906, 11 A. M., given 0.5 c.c. suspension. 6 P. M., very sick; June 28, 9 A. M., brighter; 12 M., l. p. small amount of thin fluid obtained; animal very weak. June 29, 8 A. M., moribund; chloroformed at 1 P. M. Autopsy: The membranes of the cord and brain were pale; little visible exudate. C. s. show leucocytes in small numbers over cord and brain; and a few intracellular diplococci. Sections of the tissues confirm these findings; the inflammatory exudate is small in quantity; no marked lesions of the nervous tissue itself are to be seen.

Control No. 2. *Macacus rhesus.* June 28, 12 M., 1.0 c.c. of same emulsion injected (in refrigerator over night). 6 P. M., monkey sick; 9 P. M., very sick; June 29, 9 A. M., died. Autopsy: The meninges were injected and contained small hæmorrhages. The fluid in the pia of the cord was increased, and turbid. The meninges of the brain and the ventricles contained similar fluid in excess; the basal meninges the largest quantity. C. s. show leucocytes and

diplococci throughout the membranes. The spinal membranes contain the largest number of free diplococci. Cultures positive. Sections of the tissues show the lesions of an acute inflammation of the meninges and of the superficial portion of the cortex of the brain.

In making the serum tests, the larger dose of emulsion was always employed. Brief protocols of the experiments follow:

Experiment 1, July 3, 1906: Medium sized *Macacus rhesus* given at 11 A. M. 1.0 c.c. emulsion of diplococcus "596" together with 1.0 c.c. of monkey antiserum No. 1. Fluid flowed from the needle before injection. July 4, 9 A. M., monkey appeared normal; active; on perch. L. p. yielded several drops of faintly turbid fluid, which showed on cover-slips free cocci, and leucocytes, some of which contained diplococci in moderate numbers staining feebly. Cultures from the fluid negative. July 5, 11 A. M., monkey apparently well. Lumbar puncture gave a small amount of clear fluid, which contained neither leucocytes nor cocci. Cultures negative. January 10, 1907, the monkey remained well.

Experiment 2, July 5, 1906: Medium-sized *Macacus rhesus* given 1.0 c.c. suspension of culture "596" at 11:45 A. M. Fluid flowed from needle before injection. At 2 P. M., the monkey was sick. L. p. gave a small quantity of rather thick, opaque fluid. One cubic centimeter of immune serum (monkey No. 2) injected rapidly. Immediately at conclusion of injection pressure symptoms of an alarming character developed. The animal was prostrated for two hours, after which it slowly got better. At 6 P. M., it responded to disturbance. At 8 A. M., next day, the monkey was up and appeared well. It remained so subsequently (January 10, 1907). The cover-slips from the lumbar puncture showed many polymorphonuclear leucocytes and a small number of lymphocytes, many extracellular and a few intracellular diplococci.

Before the next experiment with the serum was made, the culture "596" was again tested for virulence.

A medium-sized *Macacus rhesus* was given 1.0 c.c. emulsion at 10:30 A. M., July 11. 10 P. M., animal sick; on bottom of cage, July 12, 9 A. M., l. p., small quantity of a thin white exudate obtained. C. s. many leucocytes and few diplococci. Died 12:30 P. M. Lived about thirty-eight hours. The lesions found at autopsy were characteristic; exudate existed over the cord and brain, the base of the latter being chiefly affected. The cortical vessels were injected. C. s. showed cocci in the inflammatory exudate of the cord and base of the brain, and fewer in the exudate of the convex meninges and the ventricles. Sections of the brain and cord show marked inflammatory lesions of the usual character. Diplococci are abundant in the exudate.

Experiment 3, July 13, 1906: Moderately large *Macacus rhesus* given at 11:15 A. M. 1.0 c.c. emulsion of Coccus "596." Although no fluid was obtained, the canal was certainly entered; 4 P. M., animal sick; 5 P. M., depressed, but still sat up. L. p. gave a small quantity of turbid fluid, but during the operation the animal collapsed; 1.0 c.c. of immune serum from monkey No. 2 injected slowly. The monkey was watched until 10 P. M.; no progress of the disease. July 14, 10 A. M., animal appeared well. No future symptoms developed up to January 10, 1907. The fluid obtained by l. p. showed many leucocytes with an occasional intracellular coccus, and extracellular cocci.

Experiment 4, July 27, 1906: At 8:45 A. M., a medium-sized *Macacus rhesus* was given one full agar slant culture "610," 18 hours old, suspended in salt solution, into spinal canal Fluid obtained before injection. At 11 A. M., 5 c.c. of antiserum from monkey No. 2, were injected into the skin of the thigh. Before the serum injection the animal was sick; it lay half down on the bottom of the cage. No immediate effect followed from the injection, but the symptoms did not progress. The next day the animal appeared well. L. p. was unsuccessful after the injection until the next day, at which time clear fluid, containing neither cocci nor leucocytes, was obtained. The control for this monkey was a much smaller and weaker monkey of the same species, which succumbed in eight hours.

I do not regard this experiment as entirely free from doubt, but as I was unable to obtain more monkeys at this time, the experiment could not be repeated then.

The series of experiments was up to this point successful, and they indicated that an antiserum to the diplococcus could prevent the development of severe symptoms from following the injection of the cultures of the diplococcus into the spinal canal, and cause arrest of the symptoms which had already set in. This series of tests is, of course, incomplete without corresponding experiments with normal serum with which they may be compared. The latter will follow. But before citing them I wish to record a failure under conditions which, in view of the foregoing results, was wholly unexpected.

Experiment 5, July 20, 1906: Medium-sized *Macacus rhesus* given at 12 M. 1.0 c.c. of emulsion of Coccus "596"; 3 P. M. 1.0 c.c. of antiserum injected intraspinally. This monkey was sick when given the serum and the symptoms progressed fairly rapidly. At 11 P. M. the animal was much prostrated and sat in the cage with head depressed. It died about 7 A. M., July 21. The autopsy showed a very unusual amount of exudation in the membranes of the cord. Cover-slip preparations showed a purulent exudate with large numbers of diplococci, all within polymorphonuclear leucocytes. The exudate in the meninges of the brain and cord showed the diplococci in the same condition of complete phagocytosis, although the number of leucocytes and diplococci was smaller. The fluid withdrawn by lumbar puncture before the serum injection contained large numbers of diplococci and very few leucocytes; only an occasional leucocyte contained diplococci. Cultures made at the autopsy from the meninges of the cord, medulla and cortex, and from the lateral ventricle were positive; those from the heart and bone marrow of the femur were negative. Examination of sections of the brain and spinal cord bear out the macroscopic appearances. The exudate is remarkably thick over the entire nervous system, and is composed exclusively, or nearly so, of leucocytes. The lateral ventricle is shown to have been dilated and to contain pus cells. The brain tissue has escaped invasion with leucocytes, and the intracortical blood vessels are free from thrombi and do not show the perivascular infiltration with pus cells which is commonly present. The spinal cord at the level of the injection shows a superficial invasion with leuco-

cytes, but the higher levels do not show this condition. The dura is, in the former locality, infiltrated with pus cells.

The control for this experiment was a smaller *rhesus* monkey. The injection was made July 19, at 2 P. M., and as symptoms had failed to develop from the small dose given, a second injection of one third culture was made at 6 P. M. At 9 P. M., animal sick; lying down, but when disturbed rose and looked distrait. July 20, 7:30 A. M., lay on bottom of cage, but on being disturbed rose and displayed marked nystagmus; resumed recumbent position. Depression increased during the morning; 12 M., died. The autopsy showed a general thin exudate in the meninges, marked chiefly over the medulla. Cover-slips showed a remarkably large number of cocci which, while chiefly within leucocytes, were abundant outside. In no other experiment was so large a number of micro-organisms seen. A suspicious circumstance was found in the appearance of short chain-like groups of cocci, 4 to 6 members long. The cocci were Gram-negative and in size like the diplococcus. Attempt at cultivation failed.

In summarizing these experiments, it may be said that by the employment of an homologous anti-diplococcus serum several monkeys were saved from death due to experimental infection with *Diplococcus intracellularis*. The conditions of the experiments were such that the inoculated monkeys could, by simultaneous injection of serum and culture be prevented from developing severe symptoms, although the diplococci persisted for a period in the spinal canal, and by separate injection of the culture, and six hours later of the serum, the already severely ill monkey could, apparently, be saved from certain death. The experiment in which the serum was used successfully by subcutaneous injection cannot be interpreted without suitable repetitions.

The tests with normal monkey serum to serve as controls for the above experiments were made so as to bring out two sets of facts. In the first place, the value of simultaneous injection of normal monkey serum and a quantity of culture which would cause death in the control animal within twenty-four hours, was studied. And in the next place, the value of the normal serum was studied in monkeys in which the dose of the culture was on the border line —that is, of such a size that certain monkeys survived and others succumbed after a greater period than twenty-four hours. As regards the second series of tests, it may be said that it appeared as if the injection of a mixture of the normal serum and the culture led, in certain cases, to the survival of the monkey after a period of illness which was sometimes severe. The first symptoms ap-

peared very soon—within one or two hours—and grew in intensity for five or six hours, after which they receded. I am, therefore, inclined to attribute to normal serum employed in this way, a certain definite protective value.

The results in the first class of experiments were different. I found that the normal serum not only failed to save the inoculated monkeys, but the injection of the mixture of culture and serum might even hasten the fatal outcome. I wish to speak with some reservations on this point, for I was greatly hampered in this entire series of tests by great difficulty in obtaining at the time a suitable number of monkeys for the experiments. The study must, indeed, be carried much further before a final answer can be given as to the availability of a serum therapy for this experimental diplococcal infection in monkeys. There follow two brief protocols relating to the use of normal serum with a certainly fatal dose of the diplococcus. Two animals of the same species—*Macacus rhesus*—of equal size were employed.

Control Monkey. At 12 o'clock noon, one cubic centimeter of a suspension of the diplococcus fatal to guinea-pigs was injected into the spinal canal. Fluid flowed from the needle before injection. At 5 P. M., the monkey was sick; 9 P. M., lay on bottom of cage, but could be roused. Next morning comatose; died at 2 P. M. Survived the inoculation 26 hours. The lesions found at autopsy were characteristic. Cover-slips showed that the diplococci had to a large extent disappeared. The cultures were negative.

Serumized Monkey. At 12 o'clock noon, one cubic centimeter of the same emulsion used in the previous experiment (in refrigerator over night) mixed with one cubic centimeter of normal monkey serum and placed at 37° C. for half an hour, was injected into the spinal canal. Fluid was obtained before the injection. No immediate effects were noted following the injection. The monkey was already sick at 3:30 P. M., the symptoms increasing with great rapidity. Death took place at 8 A. M. the next morning. Survived 22 hours. The autopsy showed vivid injection of the meningeal vessels, and many small hæmorrhages over the cortical convolutions. Cover-slips showed a rich emigration of leucocytes and almost total disappearance of the diplococci. The cultures remained sterile.

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I have no desire to attempt to apply, at this time, the results given here of the experiments with the various sera on guinea-pigs and monkeys to human beings the subjects of cerebro-spinal meningitis. The experimental results with the antisera were not sufficiently constant and striking to make this mode of treatment of human cases of cerebro-spinal meningitis of very hopeful au-

gury. On the other hand, it is not improbable that more active antisera, using appropriate means of immunization, may be produced. Possibly, such antisera may prove of value in the treatment by direct spinal inoculation, possibly even by intravenous or subcutaneous injection, in this hopeless disease. The evident disadvantages to which the human patient must always be subject, as compared with the animals used for experiments, arise from the difficulty often encountered of estimating exactly the duration of the disease, and applying the remedy at its most favorable stage. On the other hand, the exceptional cases only in man run so rapid a course, attended with such profound symptoms of intoxication, as are regularly seen in the inoculation disease in animals. The slower and more measured progression of the infection in human beings may, indeed, be a favorable circumstance, provided the treatment can be applied in the early stages and before too severe structural changes have taken place in the nervous system. The fact that normal serum exercises a certain degree of protection might possibly be taken advantage of in cases of human infection. It would, of course, be practicable to obtain normal human serum for such injections. This subject is one which, in view of the gravity of cerebro-spinal meningitis in man and the absence of any efficient therapeutic measure against it, would seem to deserve consideration.

A CONTRIBUTION TO THE BACTERIOLOGY OF RHEUMATIC FEVER.

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PLATE VI.

Since Wasserman in 1899, experimenting with a diplococcus which he had isolated from a case of chorea, produced what he regarded as typical rheumatic fever in a series of eighty rabbits, much has been written for any against his view.

I do not propose in this paper to, in any way, review the position or to criticize the work of other observers. The bacterial origin of rheumatic fever seems to need no defence. The clinical features, the epidemic nature and the irregular periodicity all bring it in line with well-known bacterial diseases. Recently I have described amyloid degeneration in four cases of long standing subacute rheumatism. These cases were very typical, pure cases of rheumatism and neither in the clinical history nor in the histological examination of the organs after death was there anything to suggest any of the ordinary causes of amyloid degeneration.

Though it is true that the causes of amyloid degeneration are not clearly understood, yet it is generally recognized that the condition is secondary to some infective agent, and that, most generally, bacterial. All the experimental evidence of Krakow, Maximow, Davidsohn and others goes to support the bacterial cause.

When, however, we come to deal with the specific bacterium of rheumatism we enter much more controversial fields. It seems to me that we can dismiss from our consideration all the organisms which have been described at various times as causal except the diplococcus of Wasserman, and that which has been isolated and described in England by Poynton and Paine,[1, 2, 3] Walker,[4] Saw,[5] and myself;[6] this bacterium is probably the same as that

described by Wasserman. By these observers this organism has been isolated from definite cases of acute rheumatism, has been cultivated outside the body, has produced in rabbits and monkeys polyarthritis, endocarditis and other manifestations of rheumatic fever; and has again been isolated from the lesions in these organs. The case at first sight seems very strong, but various objections have been lodged, and it is with these that I propose dealing here.

The organism isolated, it has been thought, is not causal, the infection being simply a terminal one. Had this objection not been raised in authoritative quarters it would have been hardly worth dealing with. Terminal bacterial infections are not by any means common; besides the organism has been isolated from patients during life and when they have not been specially ill. Again, if we get a terminal bacterial infection, we expect it to be general and would expect to find the causal organism in the blood. In the three cases which I have examined after death (two of them only a few hours after), culture tubes inoculated from the blood remained sterile, and cultures of the organism were got from isolated areas only in the synovial membranes.

Acute rheumatism, it is claimed, is simply an attenuated pyæmia, and the organism isolated is an ordinary Streptococcus pyogenes. This objection has been supported by many eminent bacteriologists and must therefore be examined in detail. Rheumatism in many respects does resemble pyæmia. Both exhibit themselves in arthritis, endocarditis, etc., but there are definite distinguishing features, and save perhaps in the early stages clinicians do not mistake the one condition for the other.

Considering the very severe symptoms, and often the rapidly fatal issue in cases of acute rheumatism it seems to me a travesty of terms to speak of it as an "attenuated pyæmia." Again in pyæmia, and especially where the organism is not very active, pus formation is the common result; with acute rheumatism pus formation is the exception. As will be seen from the subjoined experiments pus was quite common in arthritis following injections of various forms of streptococci, whereas in the arthritis following inoculation with *Micrococcus rheumaticus* pus was not got in a single case.

For purposes of comparison I have examined and carried out inoculation experiments with twelve strains of streptococci and three of the special rheumatic organism:

Streptococcus 1 was isolated from a case of cellulitis; *2*, from the pus in the knee joint in a case of pyæmia; *3*, from pus in the mastoid cells in a case of middle ear disease; *4*, from a similar case; *5*, from a pyæmic abscess in the region of the appendix following an operation on a septic knee joint; *6*, from the throat in a case of measles; *7*, from the throat of a patient with scarlet fever; *8* and *9*, from separate cases of diphtheria; *10*, from a case of measles; *11*, from an acute tonsillitis; and *12*, from a case of diphtheria.

Micrococcus rheumaticus was isolated from three cases of definite acute rheumatism. These organisms were all identical in their cultural characters, but as the majority of the inoculations were carried out with one strain only, it is taken as the standard throughout the paper.

MICROCOCCUS RHEUMATICUS.

Micrococcus rheumaticus was isolated from a case of acute rheumatism on December 9, 1904, and since that time it had been subcultured frequently, often, however, intervals of two months elapsing between the times of subcultivation. The history of the case and the results of the inoculation experiments have already been published[7] and need not be further referred to here.

Morphological Characters and Staining Reaction.—It may be stated as a general rule that the coccus we have isolated, and which for convenience we call *Micrococcus rheumaticus* or *Streptococcus rheumaticus* is in its morphological characters and staining reactions indistinguishable from strains of *Streptococcus pyogenes.*

Cultural Characters.—Micrococcus rheumaticus grows quite readily at the room temperature. The growth on gelatine at the room temperature is very definite in twenty-four hours, and much more copious than the growth of any of the varieties of streptococci which were used. The acid production is extremely marked in any of the ordinary media, but some of the strains of streptococci used gave quite as marked an acid reaction, and as will be seen in the tables below the acid production and the reactions in the various sugars gave no help in distinguishing the one class of organism from the other.

The only definite and very distinctive reaction was the production of acid and precipitation of the bile salts by *Micrococcus*

rheumaticus in McConkey's bile salt lactose broth. No reaction was got in this medium with any of the strains of streptococci used. This difference was so marked that the observations were repeated with exactly similar results. The vitality of the organism outside the body I have referred to in a previous publication, but this was most strikingly illustrated during these investigations. Constant subculture was necessary to keep the streptococci alive, and three of the strains were lost during the course of the investigations. With *Micrococcus rheumaticus* there was not the slightest difficulty; several months could elapse and quite active subcultures could be obtained both in blood agar and in ordinary agar.

	Raffinose.			Inulin.			Saccharose.			Salacin.			Mannite.		
	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3
Mic. rh.	o	x	x	o	o	o	o	(x)	x	x	x	x	o	o	o
Strepto. I.	o	o	o	o	o	o	o	o	o	o	o	(x)	o	o	o
" II.	o	o	o	o	o	o	o	x	x	o	o	o	o	o	o
" III.	o	o	o	o	o	o	o	o	o	o	o	x	o	o	o
" IV.	o	o	o	o	o	o	o	x	x	o	o	o	o	o	o
" V.	o	o	o	o	o	o	o	x	x	o	o	o	o	o	(x)
" VI.	o	o	o	o	o	x	o	o	o	o	o	o	o	o	o
" VII.	o	x	x	o	o	x	o	(x)	x	o	o	o	o	o	o
" VIII.	o	x	x	o	o	o	o	o	x	o	x	x	o	o	o
" IX.	o	x	x	o	o	(x)	o	x	x	o	x	(x)	o	o	o
" X.	x	x	x	o	o	(x)	o	x	x	o	o	x	o	o	o
" XI.	o	o	o	o	o	o	o	o	(x)	o	o	o	o	o	o
" XII.	o	x	x	o	o	o	o	x	x	o	x	x	o	o	o

x indicates acid change; o indicates a negative result. Brackets ( ) indicate feeble development of the change.

	Milk.			Litmus Milk.			Taurocholate Broth.			Neutral Red.		
	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys. 2	Dys. 3
Mic. rh.	o	xc	xc	x	xc	xc	x	x	x	o	o	x
Strepto. I.	o	o	o	o	o	(x)	o	o	o	x	x	x
" II.	o	o	o	o	(x)	(x)	o	o	o	o	o	o
" III.	o	o	o	o	x	o	o	o	o	o	o	o
" IV.	o	o	o	o	o	o	o	o	o	o	o	o
" V.	o	o	o	(x)	(x)	(x)	o	o	o	(x)	(x)	(x)
" VI.	o	(xc)	(xc)	x	x	x	o	o	o	o	o	o
" VII.	o	xc	xc	x	xc	xc	o	o	o	o	o	x
" VIII.	o	o	o	x	x	x	o	o	o	o	x	x
" IX.	o	xc	xc	x	xc	xc	o	o	o	o	o	x
" X.	o	xc	xc	xc	xc	xc	o	o	o	o	o	x
" XI.	o	o	o	x	x	x	o	o	o	o	o	x
" XII.	o	o	o	x	x	xc	o	o	o	o	o	o

Brackets ( ) indicate feeble development of the respective change. c indicates coagulation.

The following tables show the cultural reactions in sugars, etc., of the strains of streptococci examined and of *Micrococcus rheumaticus.*

	Glucose Broth.			Lactose Broth.			Maltose Broth.		
	Hrs. 24	Dys. 2	Dys. 3	Hrs. 24	Dys 2	Dys. 3	Hrs 24	Dys. 2	Dys. 3
Mic. rh.	x	x	x	x	x	x	x	x	x
Strepto. I.	x	x	x	(x)	x	x	x	x	x
" II.	x	x	x	x	x	x	x	x	x
" III.	No growth.			No growth.			No growth.		
" IV.	x	x	x	(x)	x	x	x	x	x
" V.	x	x	x	x	x	x	x	x	x
" VI.	x	x	x	x	x	x	x	x	x
" VII.	x	x	x	x	x	x	x	x	x
" VIII.	x	x	x	x	x	x	x	x	x
" IX.	x	x	x	(x)	x	x	0	x	x
" X.	x	x	x	x	x	x	x	x	x
" XI.	x	x	x	x	x	x	(x)	x	x
" XII.	x	x	x	x	x	x	x	x	x

INOCULATION WITH STREPTOCOCCUS.

Cultures for these experiments were made on ordinary sloped agar tubes and an emulsion was made in 0.85 per cent. saline solution immediately before inoculation. It has not been deemed necessary to deal in detail with the microscopic examination of the various organs, though this has been carried out in a considerable number of cases.

The tables appended give the main results necessary for comparative purposes.

In this table lesions such as are common to any septic infection, *e. g.*, cloudy swelling, etc., will not be referred to.

Organism.	Rabbit.	Date and Site of Injection	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Streptococcus 1.	1	17 Jan., 1906, intravenous.	Half agar tube.	Killed, 5 Feb.	Swelling left wrist, 22 Jan.; fluctuation in left and right wrists with redness of skin, 30 Jan.	Purulent; in right and left wrists.	Nil.	Nil.
	2	17 Jan., 1906, right knee joint.	Half agar tube.	Killed, 5 Feb.	Tenderness, swelling, fluctuation in right knee.	Purulent; in right knee with infiltration with pus of tissues round.	Nil.	Nil.
	3	26 Feb. to 2 Apr., intraperitoneally.	Two loops to one agar tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.
	4	4 June, intravenous.	One agar tube.	Killed, 11 July.	Ill for two days.	Nil.	Nil.	Nil.
	5	4 June, intravenous.	Two agar tubes.	Killed, 11 July.	"	Nil.	Nil.	Nil.
Streptococcus 2.	1	24 Jan., intravenous.	Half tube.	Died, 4 Feb.	31 Jan., very ill, no evidence of joint affection.	Nil.	Nil.	Septicaemia; cultures from heart blood.
	2	24 Jan., intravenous.	Half tube.	Died, 16 Feb.		Nil.	Nil.	Heart very much dilated; septicaemia.
	3	26 Feb. to 2 Apr. 7 injections intraperitoneally.	Two loops to one tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.
	4	5 June, intravenous.	Half tube.	Died, 10 June.	—	—	—	Septicaemia; (organisms in blood).

Organism.	Rabbit	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Streptococcus 3.	1	17 Jan., subcutaneous.	One tube.	Killed, 2 Apr.	Pus formed at site of injection.	Nil.	Nil.	Nil.
	2	17 Jan., knee joint.	Half agar tube.	Killed, 2 Apr.	Swelling, tenderness and fluctuations in injected joint.	At death joint dislocated and fixed.	Nil.	Nil.
	Same rabbit (2)	3 Mar., intraperitoneally.	One agar tube.	—	Joint not altered by this injection.	—	—	—
	3	24 Jan., intravenously.	Half agar tube.	Killed, 2 Feb.	Lameness, swelling in right wrist, 30 Jan.; 31 Jan., fluctuation.	Purulent; right wrist.	Nil.	Nil.
	4	7 injections, 26 Feb. to 22 Mar. intraperitoneally.	Two loops to one tube.	Died, 23 Mar.	—	—	—	Septicaemia; streptococci in general circulation.
	5	4 June, intravenously.	One tube.	Killed, 27 June.	Nil.	Nil.	Nil.	Nil.
	6	4 June, intravenously.	One tube.	Killed, 27 June.	Nil.	Nil.	Nil.	Nil.
Streptococcus 4.	1	17 Jan., subcutaneous.	One tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.
	2	17 Jan., knee joint.	Half tube.	Killed, 15 Feb.	Swelling and fluctuation in injected knee.	Purulent; knee; erosion of bone; infiltration of tissues round knee.	Nil.	Nil.
	3	24 Jan., intravenously.	One tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.

Organism.	Rabbit.	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Streptococcus 4.	4	26 Feb. to 2 Apr., 7 injections intraperitoneally.	Two loops to one tube.	Killed, 2 Apr.	Nil.	Nil.	Nil.	Nil.
	5	30 May, intravenously.	One tube.	Killed, 16 June.	6 June, lameness left fore limb.	Purulent; left wrist.	Nil.	Nil.
	6	30 May, intravenously.	Two tubes.	Killed, 16 June.	6 June, tenderness and swelling left wrist; 12 June, fluctuation.	Purulent; left wrist.	Nil.	Nil.
Streptococcus 5.	1	16 Feb., intravenously.	Fourth tube.	Killed, 22 Feb.	17 Feb., swelling and pain in right wrist; 22 Feb., fluctuation, pain and swelling in left wrist.	Purulent; right wrist; synovial membranes in left wrist congested; no pus.	Nil.	Nil.
	2	26 Feb. to 2 Apr., 5 injections intraperitoneally.	Two loops to one tube.	Killed, 5 Apr.	Nil.	Nil.	Nil.	Nil.
	3	5 June, intravenous.	One and one-half tubes.	Died, 7 June.	Nil.	Nil.	Nil.	Septicaemia; organisms in blood.
	4	5 June, intravenous.	One and one-half tubes.	Killed, 16 June.	10 June, lame left fore limb; 12 June, fluctaution in left wrist.	Purulent; left wrist.	Nil.	Nil.
Streptococcus 6.	1	8 May, subcutaneous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.

Organism	Rabbit.	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Streptococcus 6.	2	16 May intravenous.	One tube.	Killed, 4 June.	Paralysis of both hind limbs, 22 May.	Nil.	Nil.	An abscess in lumbar region of the spinal canal pressing on the spinal cord.
		This organism died before further experiments could be made with it.						
Streptococcus 7.	1	16 May, intravenous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
	2	27 June, intravenous.	One tube.	Killed, 11 July.	Nil.	Nil.	Nil.	Nil.
	3	27 June, intravenous.	Two tubes.	Killed, 11 July.	Nil.	Nil.	Nil.	Nil.
	4	15 June to 10 July, subcutaneous and intraperitoneal; 5 injections.	Half to one tube.	Killed, 24 July.	Nil.	Nil.	Nil.	Pus at the site of subcutaneous injection.
Streptococcus 8.	1	8 May, intravenous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
	2	16 May, intravenous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
	3	29 May, intravenous.	Two tubes.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
		The organism died before further experiments could be made.						
Streptococcus 9.	1	16 May, intravenous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.

Organism.	Rabbit.	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Streptococcus 9.	2	12 July, intravenous.	Three tubes.	Killed, 17 July.	Nil.	Nil.	Nil.	Nil.
	3	15 June to 10 July, subcutaneous and intravenously 5 injections.	Half to one tube.	Killed, 24 July.	Nil.	Nil.	Nil.	Pus at the site of subcutaneous inoculation.
Streptococcus 10.	1	16 May, intravenous.	One tube.	—	—	—	—	—
	2	25 May, intravenous.	Two tubes.	Died, 5 June.	Paralysis of both hind limbs.	Nil.	Aortic endocarditis; vegetations large; ulceration of valves (ulcerative endocarditis).	Abscess in spinal column pressing on lower part of spinal cord; pyaemic abscesses in kidney.
	3	18 June, intravenous.	One tube.	Killed, 12 July.	Nil.	Nil.	Nil.	Nil.
	4	26 June, intravenous.	One tube.	Killed, 11 July.	Nil.	Nil.	Nil.	Nil.
	5	15 June to 10 July, subcutaneous and intraperitoneally; 5 injections.	Half to one tube.	Killed, 24 July.	Nil.	Nil.	Nil.	Pus at the site of subcutaneous inoculation.

Organism.	Rabbit	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Streptococcus 11.	1	16 May, intravenous; and 15 May to 10 July, subcutaneously and intraperitoneally.	One tube. Half to one tube.	Killed, 17 July.	15 June, paralysis of both hind limbs, from which it completely recovered; abscess at site of subcutaneous injection.	Nil.	Nil.	Nil.
	2	25 May, intravenous.	Two tubes.	Died, 1 June.	27 May, swelling and tenderness left wrist.	Purulent; left wrist.	Nil.	Nil.
	3	18 June, intravenous.	One tube.	Killed, 25 June.	21 June, lameness left fore limb; swelling of left wrist with considerable oedema.	Purulent; left wrist.	Nil.	Nil.
Streptococcus 12.	1	16 May, intravenous.	One tube.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
	2	20 May, intravenous.	Two tubes.	Killed, 3 July.	Nil.	Nil.	Nil.	Nil.
	3	28 June, intravenous.	One tube.	Killed, 12 July.	Nil.	Nil.	Nil.	Nil.
	4	15 June to 10 July, subcutaneous and intraperitoneal, 5 injections.	Half to one tube.	Killed, 24 July.	Nil.	Nil.	Nil.	Abscess at site of subcutaneous inoculation.

Summary of Inoculations with Streptococcus.

Intravenous	34
Intraperitoneal	7
Subcutaneous	3
Both intraperitoneal and subcutaneous	4
Total	48
Number of deaths	7 or 14.5%
Number of animals with arthritis	9 or 18.7%
Number of animals with endocarditis	1 or 2.0%

In all cases the arthritis was definitely purulent. In animals injected subcutaneously pus formed at the site of injection.

In the single experiment with endocarditis, there was distinct ulceration of the valves and the organisms were invading the adjacent muscle of the heart. There were pyæmic abscesses in the kidney, and an abscess in the lower dorsal vertebra. This lesion was evidently pyæmic endocarditis, a condition which everyone admits may occur in the course of a septic infection whatever be the organism present.

INOCULATION WITH MICROCOCCUS RHEUMATICUS.

Rabbit 1.—Inoculated in the right knee on November 8, 1905; was killed on March 26, 1906. There was stiffness about the right knee, but otherwise the animal appeared to be in perfect health. On March 8 it received an intraperitoneal injection of one half tube of culture. The following day the knee was swollen and painful, but in four days the acute symptoms had passed off. It was reinoculated in the peritoneal cavity on March 15 and 22, and on each occasion the acute symptoms reappeared and subsided again. At the post-mortem examination the joint contained a very small quantity of viscid exudation, which on examination was mainly composed of large mononucleated cells. Polymorphonuclear leucocytes were present but were very few in numbers. There was considerable destruction of cartilage with numerous small superficial erosions of the bone. No organisms of any kind were found.

There was a small recent vegetation on one of the aortic cusps. No cultivations were obtained from it. The other organs showed nothing apart from the usual toxic changes.

Rabbit 2.—Inoculated on March 29 with two tubes intravenously. April 2 the animal was lame in the right fore limb. No swelling could be made out. The lameness became less, but the animal was killed on April 6. There was well-marked aortic endocarditis, the vegetations being about a sixteenth of an inch in diameter, and pure cultures of the organism were obtained from these. With the exception of very slight injection in the synovial membranes of the left elbow nothing abnormal could be made out in any of the joints.

Rabbit 3.—Inoculated intravenously with one tube of culture on March 29.

1906; showed lameness in the left fore limb on April 5, and was killed the following day. At the post-mortem examination there was very evident congestion in the synovial membranes in the left elbow. There was also a slight amount of congestion in the left hip joint. Tubes were inoculated from portions of the synovial membranes in the congested joints but no growth was obtained. There was no endocarditis.

Rabbit 4.—Inoculated intravenously with one tube of culture on May 24, 1906; showed no symptoms of any kind during life. It was killed on June 12, but nothing pathological was made out. There was no arthritis and no endocarditis.

Rabbit 5.—Inoculated intravenously with two tubes of cultures on May 24; showed no symptoms during life. It was killed on June 12. The mitral and tricuspid segments were distinctly thickened and though no distinct vegetations could be made out there was slight nodular thickening along the free edge of the tricuspid segments. There was a firm fibrous nodule about an eighth of an inch in diameter in the septum between the ventricles. On microscopic examination this showed well-marked interstitial myocarditis. In the kidneys were some small pin-point whitish areas seen under the capsule. On section these areas extended in a wedge into the kidney substance, some of them passing almost to the hilus. On microscopic examination they were seen to be composed of masses of small lymphoid cells, with very slight development of fibrous tissue.

These interstitial charges in the heart and kidney may have been accidental and independent of the injection, but in none of the other animals examined during this investigation was the same heart condition seen. The kidney lesion was observed in three animals and these had been inoculated with Micrococcus rheumaticus. In some recent experiments by Dr. Henry Wade of Edinburgh a number of dogs were inoculated with the well-known infective granuloma of the dog. In the great majority of these experiments the kidneys showed interstitial nephritis, and in the early stages the appearance was identical with that seen in these rabbits. The interstitial changes in the kidney of the dog are undoubtedly toxic in origin, and I am inclined to attribute the changes I have described also to toxines.

Rabbit 6.—Inoculated intravenously on May 30 with one tube of culture. No symptoms developed. The rabbit was killed on June 22. There was no evidence of arthritis. On the tricuspid segments there were some very recent, fairly firm, but rather doubtful vegetations. No organisms were found. There was marked œdema of the aortic segments, but no trace of endocarditis. On microscopic examination it was difficult to determine whether the deposit on the tricuspid segments was not merely blood clot.

Rabbit 7.—Inoculated intravenously on May 30 with two tubes of culture. No symptoms developed. The rabbit was killed on June 22. There was no evidence of arthritis or of endocarditis. In both kidneys there were numerous small dark wedge shaped areas depressed slightly below the surface. The naked eye appearance suggested infarctions. On microscopic examination the area examined was found to be composed of masses of small lymphoid-like cells, and was identical with the areas seen in Rabbit 5. No bacteria could be found in these areas.

Rabbit 8.—Inoculated intravenously on June 5 with three agar tubes of the culture. No symptoms developed, and the rabbit was killed on June 22. There was no arthritis, but on the anterior mitral valve segment there was a small recent vegetation. No culture tubes were inoculated from this vegetation, but on microscopic examination a few organisms, practically unaltered in appearance, were found just at the junction of the vegetation with the tissue of the valve.

Rabbit 9.—A young rabbit of 600 grams weight was inoculated intravenously on June 13 with the culture from four agar tubes. On June 17 the rabbit looked very ill, and could not be got to move about. It was impossible to decide as to the presence or absence of arthritis. The animal died on June 18 and at the post mortem examination a slight amount of serous fluid was found in both elbows, but there was no congestion of the synovial membranes. On microscopic examination the fluid contained some mononucleated cells, but hardly any polymorphonuclear leucocytes. No bacteria were detected. Cultures were negative. The synovial membranes of both knees were very markedly congested, and there was very evident dilatation of the vessels along the inner side of the condyles of both femora. There was a slight amount of clear fluid in the joint cavities. On microscopic examination there were a fair number of polymorphonuclear cells, but the large proportion of the cells were mononucleated. These were markedly vacuolated and showed abundant phagocytosis of polymorphonuclear cells.

Cultures in milk broth, made by inoculation with small pieces of the membranes, gave pure cultures of the organism, whereas those inoculated with the fluid in the joint remained sterile.

No vegetations were made out. Microscopic examinations of the heart blood showed no organisms, and cultures from it remained sterile. Microscopic examination of the synovial membranes showed irregular scattered areas of polymorphonuclear leucocytes and in these areas only were the organisms found (Fig. 1). This experiment is of extreme interest, for it seems to me to be in line with many cases of rheumatism. The organism is localized and produces its main effect by a toxin; it also proves that failure to obtain the organism from the blood or even from the joint exudation is no proof that the organism is not present.

Rabbit 10.—Inoculated intravenously with four tubes of culture on June 25; showed very definite lameness in the right fore limb on June 28, and on June 29 in the right hind limb. The rabbit recovered completely and was killed on July 18. Nothing abnormal could be made out on naked eye examination. Microscopic examination is not yet completed.

Rabbit 11.—Inoculated intravenously with four tubes of culture on June 25; showed very slight lameness in the right fore limb on June 28. No tenderness or swelling could be made out. The animal was quite well the following day. It was killed on July 18. Microscopic examination is not yet complete. No organisms were obtained in culture from the joints.

Rabbit 12.—Inoculated intravenously on June 26 with the growth on five culture tubes. On June 29 there was very definite lameness in the right hind limb. On July 1 there was very distinct improvement in the condition. On July 2 the animal was dragging the left hind limb also, and on July 18 when

Organism.	Rabbit.	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis	Other Lesions.
Micrococcus rheumaticus.	1	8 Nov., 1905 right knee joint.	A few loopsful.	26 Mar., 1906, killed.	Swelling and tenderness later, stiffness in right knee.	Non purulent with some destructive changes.	Very recent aortic.	
	2	29 Mar., intravenously.	Two tubes.	Killed, 8 Apr.	Lameness right fore limb.	Slight injection in synovial membrane of right elbow.	Well marked aortic.	
	3	29 Mar., intravenously.	One tube.	Killed, 6 Apr.	Lameness left fore limb.	Congestion of synovial membrane in left elbow.	Nil.	
	4	24 May, intravenously.	One tube.	Killed, 12 June.	Nil.	Nil.	Nil.	Nil.
	5	24 May, intravenously.	Two tubes.	Killed, 12 June.	Nil.	Nil.	Thickening of mitral and tricuspid segments.	Interstitial myocarditis; acute interstitial nephritis.
	6	30 May, intravenously.	One tube.	Killed, 22 June.	Nil.	Nil.	Doubtful vegetations on tricuspid and aortic segments.	Nil.
	7	30 May, intravenously.	Two tubes.	Killed, 22 June.	Nil.	Nil.	Nil.	Acute interstitial nephritis.
	8	5 June, intravenously.	Three tubes.	Killed, 22 June.	Nil	Nil.	Recent vegetation on mitral segment.	Nil.
	9	13 June, intravenously.	Four tubes.	Died, 18 June.	Indefinite, but animal acutely ill.	Acute in both knees.	Nil.	Nil.

Organism.	Rabbit.	Date and Site of Injection.	Quantity of Injection.	Date of Death.	Symptoms Noted During Life.	Post Mortem.		
						Arthritis.	Endocarditis.	Other Lesions.
Micrococcus rheumaticus.	10	25 June, intravenously.	Four tubes.	Killed, 18 July.	Lameness in right fore and right hind limb (recovered).	Nil.	Nil.	Examination not completed.
	11	25 June, intravenously.	Four tubes.	Killed, 18 July.	Lameness in right fore limb (recovered).	Nil.	Nil.	Examination not completed.
	12	26 June, intravenously.	Five tubes.	Killed, 18 July.	Lameness right hind limb; partial recovery.	Congestion of synovial membranes on both hips.	Nil.	Acute interstitial nephritis.
	13	10 July, intravenously.	Four tubes.	Killed, 13 July.	Indefinite; animal acutely ill.	Right knee.	Nil.	Septicaemia.
	14	10 July, intravenously.	Two tubes.	Died, 13 July.	Indefinite, but acutely ill.	Both knee joints.	Minute vegetations in mitral segments.	Septicaemia.
	15	10 July, subcutaneously.	Five c.c. broth culture.	Killed, 24 July.	Nil.	Nil.	Nil.	Nil.

it was killed, both hind limbs were weak and they appeared to be paralyzed. When the animal was allowed to run about it could move its limbs fairly well.

At the post mortem examination there was definite congestion of the ligamentum teres and synovial membranes generally in both hips. Nothing abnormal was made out in any of the other joints. There was no endocarditis. In the kidney there were several small wedge-shaped areas resembling infarctions. These were dark red and were depressed slightly below the surface of the organ. Microscopically these areas corresponded with those described in Rabbits 5 and 7.

Rabbit 13.—Inoculated intravenously on July 10 with the growth from four culture tubes. The following day the animal was evidently ill, but no arthritis could be made out.

On July 13 it was killed. There was slight injection of the synovial membrane in the right knee, and microscopic examination showed the presence of polymorphonuclear and mononucleated cells and also bacteria similar to Micrococcus rheumaticus.

Cultures from the heart blood gave a pure growth of the micrococcus. There were no vegetations.

Rabbit 14.—Inoculated intravenously on July 10 with the growth from two agar tubes. On July 11 it was very ill, and was found dead in its cage on July 13. There was congestion in the synovial membranes of both knee joints, and from the right pure cultures of the micrococcus were obtained. A pure culture was also obtained from the blood in the heart. There were numerous minute vegetations along the free margin of the mitral valve segments. On microscopic examination the vegetations were very definite and there was infiltration of the tissue of the valve with polymorphonucleated and mononucleated cells.

Rabbit 15.—Inoculated subcutaneously with 5 c.c. of a broth culture of Micrococcus rheumaticus on July 10, 1906. No pus developed at the site of inoculation. The rabbit was killed on July 24. No pathological lesions were detected.

Summary of Inoculations with Micrococcus Rheumaticus:

Intravenous	13
Into knee joint	1
Subcutaneous	1
Total	15
Number of deaths	2 or 13.3%
Number of animals with arthritis	9 or 60.0%
Number of animals with endocarditis (including one doubtful case)	5 or 33.3%

In all the experiments the arthritis was non-purulent, and the knee joints were frequently affected. Recovery or improvement took place if the animal was allowed to live. I would also call special attention to the presence of acute interstitial nephritis and myocarditis in the rheumatic cases. These were not seen in any

of the streptococcal cases. Their significance I am not yet in a position to state.

AGGLUTINATION.

By this means also differentiation is made out between the various forms of streptococci and *Micrococcus rheumaticus*. These reactions were all done the same day with similar solutions, and the results were checked by the independent observations of Dr. J. W. Dawson, to whom I am indebted for very much help during the course of these investigations.

The Agglutination was tested in sedimentation tubes. Dilution was 1 in 30.
Reaction xx = Definite Clumping.

I. (*Control*) *Emulsions alone:* In peptone broth of the separate organisms gave the following reactions:

Micrococcus rheumaticus	A few clumps.
Streptococcus I	No clumps or very few.
Streptococcus II	No clumps or very few.
Streptococcus III	No clumps or very few.
Streptococcus IV	No clumps or very few.
Streptococcus V	A few clumps.

II. (*Control*) *Normal Rabbit Serum:* Plus emulsion in peptone broth of the separate organisms gave the following reactions:

Micrococcus rheumaticus	Hardly any change.
Streptococcus I	Nil.
Streptococcus II	Nil.
Streptococcus III	Nil.
Streptococcus IV	Nil.
Streptococcus V	A few clumps but not nearly so marked as in immune serum.

III. Serum of Rabbit Immunized with Streptococcus I.

Plus emulsion in peptone broth of M.rh.	x ?	Very few clumps
Plus emulsion in peptone broth of Strepto. I	xxx	
Plus emulsion in peptone broth of Strepto. II	x	
Plus emulsion in peptone broth of Strepto. III	xxx	
Plus emulsion in peptone broth of Strepto. IV	xx	
Plus emulsion in peptone broth of Strepto. V	xxx	

IV. Serum of Rabbit Immunized with Streptococcus II.

Plus emulsion in peptone broth of M.rh.	x ?
Plus emulsion in peptone broth of Strepto. I	xxx
Plus emulsion in peptone broth of Strepto. II	xx
Plus emulsion in peptone broth of Strepto. III	xx
Plus emulsion in peptone broth of Strepto. IV	xx
Plus emulsion in peptone broth of Strepto. V	xxxx

V. Serum of Rabbit Immunized with Streptococcus III.

Plus emulsion in peptone broth of M.rh.	x	
Plus emulsion in peptone broth of Strepto. I	xxx	
Plus emulsion in peptone broth of Strepto. II	xx	
Plus emulsion in peptone broth of Strepto. III	xxxx	
Plus emulsion in peptone broth of Strepto. IV	xx	
Plus emulsion in peptone broth of Strepto. V	xxx	

VI. Serum of Rabbit Immunized with Streptococcus IV.

Plus emulsion in peptone broth of M.rh.	x	Very slight; one large clump at bottom of tube.
Plus emulsion in peptone broth of Strepto. I	xx	
Plus emulsion in peptone broth of Strepto. II	x	
Plus emulsion in peptone broth of Strepto. III	xx	
Plus emulsion in peptone broth of Strepto. IV	xx	But some very large clumps.
Plus emulsion in peptone broth of Strepto. V	xx	

VII. Serum of Rabbit Immunized with Streptococcus V.

Plus emulsion in peptone broth of M.rh.	Nil.	
Plus emulsion in peptone broth of Strepto. I	xx	
Plus emulsion in peptone broth of Strepto. II	x	
Plus emulsion in peptone broth of Strepto. III	xxx	
Plus emulsion in peptone broth of Strepto. IV	x	Very slight.
Plus emulsion in peptone broth of Strepto. V	x	

VIII. Serum of Rabbit Immunized with Micrococcus rheumaticus.

Plus emulsion in peptone broth of M.rh.	xx	Big clumps; bigger than with Strepto. I.
Plus emulsion in peptone broth of Strepto. I	xx	
Plus emulsion in peptone broth of Strepto. II	Nil.	
Plus emulsion in peptone broth of Strepto. III	x	
Plus emulsion in peptone broth of Strepto. IV	Nil.	
Plus emulsion in peptone broth of Strepto. V	x ?	

The absence of the organisms from the blood and joint exudations during life. Poynton and Paine and Walker and Beaton record quite frequently successful cultivations from the blood and the exudates in the joints during life. Philipp,[8] in twenty-four cases of acute articular rheumatism, attempted cultivations twenty-one times from the blood and six times from the joint exudate, and twice from the blood and twice from the joints in chronic rheumatism. No bacteria were cultivated, though various kinds of media were used. Cole[9] reports that for the last three years, in prac-

tically all cases of acute rheumatism treated in the Johns Hopkins Hospital, routine cultures have been made from the blood and from the joints whenever any effusion was present. All the cultures were negative.

My own experience in this connection has been very limited. I have examined three cases of acute rheumatism—and all of them post-mortem. In all of them tubes inoculated from the blood remained sterile. In only one case cultures were made from the exudate in the joint, and these also were negative. In the three cases, however, the organism was grown from pieces of the synovial membrane. Several of the tubes inoculated with pieces of synovial membrane also remained sterile.

The results of my culture experiments, and also of microscopic examination, indicate that the organism may be missed altogether in cases where the arthritis is the principal manifestation of the disease, unless several different areas of the synovial membranes are examined.

No doubt in some cases, and especially in severe attacks and those with vegetative endocarditis, the organisms may be found in the blood at certain stages of the disease, but in most of the ordinary cases the organisms appear to be localized and probably produce their results by a toxin. Unless these localized areas are examined there is no possibility of getting cultivations.

Rabbit 9 is of extreme interest in this connection as in it has been reproduced a condition which is the common one in acute rheumatism in the human subject, *i. e.,* a localized bacterial infection in the synovial membranes and secondary effects the result of a toxine. By definite experiment, I have thus shown a complete picture of a case of infection with *Micrococcus rheumaticus* where joint exudation and blood are sterile. Unfortunately, I have, through want of time, not yet been able to repeat this experiment.

Further, the "interstitial nephritis" seen in several of the rabbits, shows the secondary results of the toxine. This also came under my notice late in the research, and has opened up a field which will require careful investigation before any definite conclusion can be drawn from it.

CONCLUSION.

The conclusions I would draw from this work are merely those stated in a former paper.

1. The results obtained by injections of streptococci are different from those produced by *Micrococcus rheumaticus*.

2. *Micrococcus rheumaticus* cannot be regarded as an attenuated streptococcus, nor acute rheumatism as an attenuated streptococcal pyæmia.

3. In uncomplicated cases of acute rheumatism the organism may not be found in the blood or in the joint exudates.

EXPLANATION OF PLATE VI.

FIGURE 1. Synovial Membrane (Rabbit 9) showing localized inflammatory areas (*a*) in which the bacteria were found.

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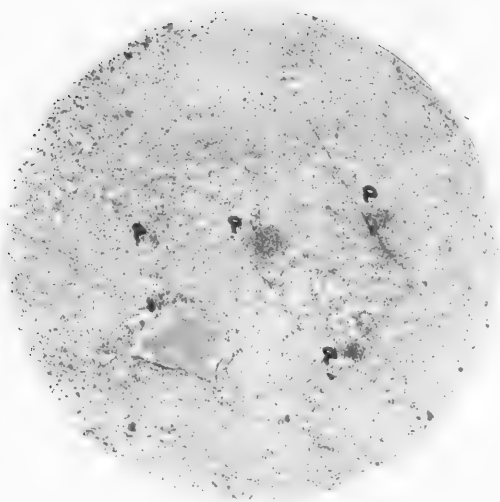

FIG. 1.

LEUCOPROTEASE AND ANTI-LEUCOPROTEASE OF MAMMALS AND OF BIRDS.

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Studies of Fr. Müller[1] and subsequent observers have shown that the polynuclear leucocytes of man and other mammals contain an enzyme which digests proteid and is particularly active in the presence of a weak alkaline reaction. One of us[2] has shown that a suspension of cells from an inflammatory exudate causes proteolysis both in an alkaline and in an acid medium, though digestion is more active in the former. It has further been possible to obtain from such cells a dry powder[3] which digests only in the presence of an alkaline reaction; this enzyme has been designated leucoprotease.

Proteolysis caused by cells of an inflammatory exudate in the presence of acid is more active in proportion to the number of large mononuclear phagocytes or macrophages which are present, while an emulsion made from the lymphatic glands which are situated near the seat of inflammation and contain such cells in immense number causes proteolysis in an acid medium and fails to digest in a neutral or alkaline solution; for convenience, this enzyme has been designated lympho-protease. Hence, each of the two types of phagocytic cells which are capable of ingesting and dissolving within their substance micro-organisms and other proteid-containing bodies is characterized by an enzyme, namely, the polynuclear leucocyte contains leucoprotease, which digests in a neutral or alkaline medium while the large mononuclear phagocyte, which is most abundant in the inflammatory exudate during the later stages of inflammation and often attacks and digests the smaller polynuclear cell, contains lymphoprotease which digests only in the presence of acid.

[1] Kossel, *Zeit. f. klin. Med.*, 1888, xiii, 149.
[2] *Jour. of Exper. Med.*, 1905, vii, 316.
[3] *Ibid.*, 1906, viii, 410.

Leucoprotease, like trypsin, digests in the presence of an alkaline reaction, while lympho-protease, like pepsin, requires an acid medium. Lymphoprotease is active in the presence of very weak hydrochloric acid (1/100 N.) but fails to act in the presence of that concentration of hydrochloric acid which is most favorable to the action of pepsin.[4] Leucoprotease, moreover, has been found to be far less active than trypsin. Further knowledge concerning the relation of the enzymes of leucocytes to the enzymes of the digestive tract is wanting.

The studies of Salkowski[5] have shown that organs kept at body temperature under conditions which prevent bacterial growth undergo self-digestion. Bondi[6] found that self-digestion of liver tissue is more active in an acid than in an alkaline medium. Hedin and Rowland[7] have shown that juice expressed from the spleen of the beef, horse, pig and sheep undergo especially active autolysis in the presence of an acid reaction. The reaction of the expressed juice is acid, but the addition of 0.1 per cent. hydrochloric acid increases proteolysis. In the presence of an alkaline reaction produced by adding from 0.2 to 0.37 per cent. of sodium bicarbonate, the degree of autolysis is diminished, but is, nevertheless, considerable. Subsequent observations[8] have shown that lymphatic glands, the kidneys and the liver contain proteolytic enzymes which are more efficient in an acid than in an alkaline medium. The juice expressed from voluntary muscle undergoes only slight autolysis which is not increased either by addition of acid or of alkali. The heart muscle contains an enzyme which resembles that of other organs and is more active in the presence of acid. Levene and Stookey[9] found that autolysis of nerve tissue and of testis is increased by acid.

Hedin[10] succeeded in separating from the spleen two enzymes, one of which, designated by him lieno-α-protease, acted in alkaline

[4] *Ibid.*, 1906, viii, 418.
[5] *Zeit. f. klin. Med.*, 1890, Suppl. zum xvii, 77.
[6] *Virchow's Archiv.* 1896, cxliv, 373.
[7] *Zeit. f. physiol. Chem.*, 1901, xxxii, 341.
[8] *Ibid.*, 1901, xxxii, 531.
[9] *Jour. Med. Research*, 1903, x, 212.
[10] *Jour. of Physiol.*, 1904, xxx, 155.

medium, while a second enzyme, which he called lieno-β-protease, acted in the presence of acid. After spleen pulp had been digested in the presence of 0.1–0.2 per cent. acetic acid, that enzyme which digested in the presence of acid was found in solution, while from the residue, after extraction with three per cent. sodium chloride, precipitation with acetic acid and subsequent neutralization, an enzyme was obtained which acted rather strongly in an alkaline medium and much less in an acid. Hedin showed that the enzymes which he isolated not only caused autolysis, by digesting the substance of the cells which contained them, but were capable of breaking down other proteids, such as fibrin, casein and coagulated blood serum.

The cells of an inflammatory exudate obtained by injecting aleuronat into the pleural cavity of the dog, unlike all of the organs which have been mentioned cause more active proteolysis in an alkaline than in an acid medium. Another tissue has been found by one of us[11] to share this property, namely, the bone-marrow. A suspension of cells from the spleen, lymphatic glands, liver or kidneys caused much more active proteolysis of heated serum with acid than with alkali, while similarly prepared suspensions of cells from the bone-marrow were far more active in an alkaline medium. It is not improbable that the enzyme which is present in large amount in the cells of the exudate rich in polynuclear leucocytes and in the tissue from which the polynuclear lucocytes arise is identical with the similar enzyme which Hedin obtained from the spleen.

The Relation of Anti-leucoprotease to the Globulins and Albumin of the Blood Serum.—Hahn[12] first showed that normal blood serum has the power of inhibiting or wholly preventing the action of trypsin. According to Landsteiner[13] this anti-enzymotic action is not possessed by the serum globulin, precipitated by half saturation with ammonium sulphate, but is present in the albumin precipitated by complete saturation with ammonium sulphate after removal of the globulin. Glaessner[14] failed to confirm this observation, main-

[11] *Jour. of Exper. Med.*, 1905, vii, 759.
[12] *Berliner klin. Woch.*, 1897, xxxiv, 499.
[13] *Cent. f. Bakt.*, 1900, xxvii, Abt. i, 357.
[14] *Hofmeister's Beiträge*, 1904, iv, 79.

taining that the euglobulin fraction precipitated by one third saturation with ammonium sulphate inhibited the action of trypsin on coagulated proteid contained in Mett's tubes, while the pseudo-globulin subsequently precipitated by half saturation exhibited little, and the albumin fraction, no anti-enzymotic action. The results obtained by Landsteiner have been confirmed by Cathcart,[15] who found anti-tryptic action with the albumin, but not with the globulin fraction. This anti-enzymotic action of the serum is destroyed by a temperature of 70° C. but that of the isolated albumin fraction is destroyed by 55° C.

That enzyme of the spleen which acts in an alkaline medium and has been designated by Hedin lieno-α-protease is inhibited by the blood serum. Hedin found that the substance in the serum of the ox which checked this enzyme was contained in the albumin and pseudo-globulin fractions, whereas, in one experiment the euglobulin fraction slightly increased, in another slightly diminished, its activity. That part of the euglobulin fraction which was precipitated by dialysis of the serum was found to increase rather than diminish proteolysis caused by the splenic enzyme.

It has been shown by one of us[16] that proteolysis in an alkaline or approximately neutral medium caused by a suspension of cells from an inflammatory exudate is prevented by small quantities of the serum of the exudate or of the serum of the blood. This anti-enzymotic action is destroyed by a temperature of 75° C. but is unaffected by heating to 70° C. during an half hour. Baer and Loeb[17] observed that the serum of the blood exerted a similar action upon the autolytic enzyme contained in the liver, but since, as they believed, this property was little, if at all, altered by heat, even at the temperature of boiling, they did not think it attributable to a true anti-enzyme. The same inhibiting action was exerted by the albumin of the serum but was wholly lacking in the globulin.

The purpose of the following experiments has been to determine primarily if the power to resist the action of the leucoprotease of the polynuclear leucocytes is common to all proteids of the blood

[15] *Jour. of Physiol.*, 1904, xxxi, 497.
[16] *Jour. of Exper. Med.*, 1905, vii, 316.
[17] *Arch. f. exper. Path. u. Phar.*, 1905, liii, 1.

serum or if this anti-body is localized in a particular fraction of the serum. The somewhat discordant results obtained with anti-bodies for other proteolytic enzymes have been cited for comparison.

To separate the globulins and albumin of the blood, the method of fractioning the serum employed by Freund and Joachim[18] has been used. The blood serum after centrifugalization was diluted with three times its volume of distilled water. A small amount of precipitate, euglobulin, was obtained by adding a saturated solution of ammonium sulphate in quantity to cause one third saturation. The precipitate was washed, dissolved in distilled water, and dialyzed; it was again precipitated by one third saturation with ammonium sulphate and again dialyzed. To the filtrate obtained after one third saturation was added a saturated solution of ammonium sulphate in quantity sufficient to cause one half saturation. The bulky precipitate thus obtained was dissolved in water, dialyzed and reprecipitated. The filtrate was completely saturated with ammonium sulphate by the addition of dry salt. A bulky precipitate, albumin, was obtained and further purified as before. The fractions of serum obtained by this method were dissolved in a volume of water approximately equal to or in some instances double that of the serum employed.

Dried and powdered leucocytes prepared by the method previously described (leucoprotease), in weighed quantities, were allowed to act at 37° C. during five days on a measured quantity of coagulated proteid (five cubic centimeters of heated serum) in the presence of the various fractions which had been isolated. Digestion took place in closely stoppered flasks; the volume of each mixture was brought to twenty-five cubic centimeters by addition of 0.85 per cent. salt solution, and one cubic centimeter of toluol was added. The amount of nitrogen in substances incoagulable by heat has been measured by the Kjeldahl method, and for the sake of comparison is given in terms of cubic centimeters of 1/10 N. sulphuric acid. Since previous experiments had shown that the anti-enzymotic activity of the serum was slightly greater when the alkalinity of the medium was increased, to each mixture in the following experiment was added 0.2 per cent. of sodium carbonate;

[18] *Zeit. f. physiol. Chem.*, 1903, xxxvi, 407.

in subsequent experiments this addition was not made. Former experiments having shown that the anti-enzymotic action of the serum is destroyed by heat, the effect of the unheated fraction was compared with that of the same fraction previously heated to 75° C. during one half hour.

20 mgr. leucoprotease+coagulated proteid+10 c.c. euglobulin	20.5 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. euglobulin heated	16.1 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. pseudo-globulin	27.2 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. pseudo-globulin heated	21.05 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. albumin	13.8 c.c.
20 mgr. leucoprotease+coagulated proteid+10 c.c. albumin heated	29.7 c.c.

In order to test the action of the various fractions in the absence of leucoprotease, quantities of pseudo-globulin and of albumin equal to those previously employed were allowed to act on heated serum under conditions similar to those just described, addition of alkali being omitted. The control represents the amount of nitrogen in uncoagulable form contained in a given mixture before digestion.

10 c.c. pseudo-globulin	+ coagulated proteid	21.1 c.c.
Control		6.95 c.c.
10 c.c. albumin	+ coagulated proteid	10.8 c.c.
Control		9.6 c.c.

The experiment demonstrates that digestion is increased when the euglobulin and pseudo-globulin fractions are added to a mixture of leucoprotease and heated serum. Digestion is inhibited on the contrary by the albumin fraction. The second half of the experiment shows that the pseudo-globulin fraction contains an active proteolytic enzyme, while the albumin fraction fails to cause noteworthy digestion.

The following experiments confirm the results of that just described. The conditions of the experiments are the same, save that euglobulin and pseudo-globulin were not separated but were precipitated together by half saturation with ammonium sulphate. The globulin fraction was dissolved in a volume of water approximately equal to that of the serum from which it was obtained. The albumin fraction was dissolved in twice its volume of water.

20 mgr. leucoprotease + coagulated proteid + 15 c.c. globulin	33.45 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. globulin heated	24.2 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. albumin	7.5 c.c.
20 mgr. leucoprotease + coagulated proteid + 15 c.c. albumin heated	18.1 c.c.

The action of the two fractions on coagulated proteid in the absence of leucoprotease was further tested.

Coagulated proteid + 15 c.c. globulin	28.1 c.c.
Control	10.3 c.c.
Coagulated proteid + 15 c.c. albumin	7.05 c.c.
Control	6.55 c.c.

The globulin and albumin fractions used in the next experiment were dissolved in volumes of water approximately equal to that of the serum from which they were obtained.

20 mgr. leucoprotease + coagulated proteid + 5 c.c. globulin	25.2 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. globulin heated	23.85 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. albumin	13.1 c.c.
20 mgr. leucoprotease + coagulated proteid + 5 c.c. albumin heated	22.6 c.c.

The same quantities of the globulin and of the albumin were allowed to act upon coagulated proteid at 37° C. during five days in the presence of an approximately neutral, alkaline, and acid reaction:

Coagulated proteid + 5 c.c. globulin	16.3 c.c.
Coagulated proteid + 5 c.c. globulin + 0.2 per cent. sodium carbonate	6.5* c.c.
Coagulated proteid + 5 c.c. globulin + 0.2 per cent. acetic acid	3.7 c.c.
Control	2.3 c.c.
Coagulated proteid + 5 c.c. albumin	2.4 c.c.
Coagulated proteid + 5 c.c. albumin + 0.2 per cent. sodium carbonate	2.55 c.c.
Coagulated proteid + 5 c.c. albumin + 0.2 per cent. acetic acid	2.15 c.c.
Control	2.4 c.c.

* Since this figure differed markedly from that obtained when the reaction of the medium remained approximately neutral (16.3 c.c.) it was suspected that an error had occurred in making the Kjeldahl determination; repetition of the test gave the figure, 13.2 c.c.

These experiments which show that the anti-enzyme for leucoprotease is present in the albumin of the serum and absent in the globulins are analogous to those of Landsteiner and of Cathcart who found anti-trypsin only in the albumin fraction of the serum. Hedin, it has been mentioned, found an anti-body for his lieno-α-protease in the albuimn fraction, but present also, according to his observations, in the pseudo-globulin.

The globulin of the serum not only possesses no anti-leucoprotease but contains a proteolytic enzyme which is active under conditions similar to those which favor the action of leucoprotease. Delezenne and Pozerski[19] had observed that serum treated with chloroform

[19] *Compt. rend. Soc. de Biol.*, 1903, lv, 327, 690, 693.

digests proteid, and believed that the serum contained an anti-body which normally held this enzyme in check. Hedin[20] showed that a weak proteolytic enzyme which digests in an alkaline medium is present in the globulin of the serum and is inhibited by an anti-body which is mainly contained in the albumin. It is not improbable that this enzyme is identical with that which is present in the polynuclear leucocytes and in the bone marrow and with the similar enzyme of the spleen. In the blood serum this enzyme is held in check by the anti-body which is precipitated with the albumin; this anti-body which in given quantity doubtless holds in check only a limited quantity of enzyme,[21] is present in the serum in excess, so that the whole serum is capable of further anti-enzymotic action.

Action of Anti-leucoprotease of Different Mammalian Species upon Leucoprotease of the Same and of Different Species.—By study of the anti-enzymotic action of sera from animals of various species upon leucoprotease derived from different animals, it was hoped that evidence concerning the identity or multiplicity of such enzymes might be obtained; for if each of two enzymes bears a specific relationship to its own serum, it is improbable that the two enzymes are identical. Glaessner believed that the anti-tryptic action of the blood serum bears a specific relation to trypsin from the same species; he found that trypsin from the ox was more strongly inhibited by ox serum and trypsin from the pig by pig's serum than by other sera. The experiments of Cathcart[22] did not confirm this view, but were not decisive.

In order to test the inhibiting action of sera obtained from a variety of species upon the leucoprotease of the dog, a measured quantity (twenty milligrams) of the dry powder prepared from leucocytes, obtained either by injecting aleuronat into the pleural cavity or turpentine into the subcutaneous tissue, was allowed to act during five days at 37° C. upon a measured quantity of coagulated proteid in the presence of various sera. In the following experiment three cubic centimeters of the serum of dog, man, and ox were employed. To determine for comparison the proteolytic activity of

[20] *Jour. of Physiol.*, 1904, xxx, 195.
[21] *Jour. of Exper. Med.*, 1906, viii, 538.
[22] *Loc. cit.*

the enzyme, twenty milligrams were allowed to act upon coagulated proteid in the presence of three cubic centimeters of the various sera previously heated to 75° C. during one half hour, in order to destroy their anti-enzymotic action. The control represented the amount of nitrogen in uncoagulable substances present in the mixtures before digestion.

	Human serum.	Dog's serum.	Ox's serum.
Control	3.35 c.c.	4.65 c.c.	3 c.c.
With 3 c.c. heated serum	25.25 c.c.	26.5 c.c.	21.8 c.c.
With 3 c.c. unheated serum	7.4 c.c.	8.7 c.c.	7.6 c.c.

The sera of man and of ox act like that of the dog, and hinder digestion caused by the dog's enzyme. It is not improbable that a maximum degree of inhibition has been caused by these sera when three cubic centimeters of each were employed. In order to test more accurately the relative activity of the different sera, smaller quantities were employed; 0.5 and 0.25 cubic centimeters of the serum of dog, cat, goat, and pigeon were added to mixtures containing twenty milligrams of leucoprotease and a measured quantity of coagulated proteid.

	Dog's serum.	Cat's serum.	Goat's serum.	Pigeon's serum.
With 0.5 c.c. serum	9.9 c.c.	4.3 c.c.	4.75 c.c.	16.8 c.c.
With 0.25 c.c. serum	10.85 c.c.	4.25 c.c.	6.4 c.c.	17.6 c.c.

The proteolysis caused by twenty milligrams of the enzyme acting on the quantity of coagulated proteid used in the above mixtures is represented by 18.2 c.c., the control being 3.0 c.c.

The serum of the cat causes more complete inhibition of the dog's enzyme than the serum of the goat, but both sera are more actively anti-enzymotic than the dog's own serum. Digestion in the presence of equal quantities of pigeon's serum is little less than that caused by the unrestrained enzyme. In the following experiment the sera of dog and rabbit were compared, the effect of increasing quantities of these sera on twenty milligrams of dog's leucoprotease being tested. Since the coagulated proteid used for digestion (five cubic centimeters of heated dog's serum) was not the same in the two series, the results are not accurately comparable, but demonstrate that the inhibiting power of the rabbit's serum is considerably greater than that for the dog.

	Dog's serum.	Rabbit's serum.
With 0.25 c.c. serum	22.15 c.c.	7.35 c.c.
With 0.5 c.c. serum	18.8 c.c.	5.35 c.c.
With 1 c.c. serum	10.6 c.c.	4.3 c.c.
With 2.5 c.c. serum	7.55 c.c.	4.9* c.c.
Without the addition of serum	24.2 c.c.	21.45 c.c.
Control	2.85 c.c.	2.85 c.c.

* This figure is larger than that obtained when a smaller quantity of rabbit's serum was used because the mixture before digestion contained more nitrogen in uncoagulable form, 2.5 c.c. of dog's serum being represented by 1.45 c.c. and 2.5 c.c. rabbit's serum by 1.75 c.c.

In the second experiment the figures obtained are comparable, the same coagulated proteid being used for digestion in all of the tests.

	Dog's serum.	Rabbit's serum.
With 0.25 c.c. serum	12.5 c.c.	6.45 c.c.
With 1.0 c.c. serum	6.5 c.c.	5.15 c.c.

Digestion caused by the enzyme used in this experiment, in the absence of serum, is represented by 20.05 c.c., the control being 4.05 c.c.

Since the enzyme of the dog's leucocytes is more markedly inhibited by the serum of man, ox, cat, goat, and rabbit than by the dog's own serum, it is necessary to determine if the corresponding enzyme of other animals bears the same relation to foreign mammalian sera. For this purpose, the attempt was made to obtain polynuclear leucocytes of the rabbit in quantities sufficient for the tests required. By injection of aleuronat into the pleural cavity of the rabbit, only a small quantity of exudate was obtained, and this exudate was so poor in cells that their suspension in salt solution completely failed to digest coagulated proteid. Injections of turpentine into the subcutaneous tissue of the rabbit caused exudation of serum and accumulation of dry friable material of opaque white color forming a layer adherent to the necrotic tissues which had been in contact with the injected turpentine. This material consisted in great part of polynuclear leucocytes held together by fibrin; typical suppuration with softening and solution of tissue was entirely lacking. The exudate was scraped from the surface of the exposed tissues and dried by the method previously described. The dry powder thus obtained was found to have weak proteolytic action when allowed to act at body temperature on heated serum. The anti-

enzymotic action of rabbit's, dog's and hen's serum was tested with this enzyme.

20 mgr. leucoprotease + coagulated proteid + 0.5 c.c. rabbit's serum	3.1 c.c.
20 mgr. leucoprotease + coagulated proteid + 0.5 c.c. dog's serum	3.7 c.c.
20 mgr. leucoprotease + coagulated proteid	4.15 c.c.
Control	2.5 c.c.

Since the amount of digestion in the experiment, even in the absence of serum, was insignificant, a larger quantity of the ferment was employed.

50 mgr. leucoprotease + coagulated proteid + 0.5 c.c. rabbit's serum	3.45 c.c.
50 mgr. leucoprotease + coagulated proteid + 0.5 c.c. dog's serum	4.05 c.c.
50 mgr. leucoprotease + coagulated proteid	7.6 c.c.
Control	2.45 c.c.

These two experiments have shown that leucoprotease derived from the rabbit bears to the two sera with which it has been tested a relation which is identical with that of dog's leucoprotease to the same sera; the enzyme from both animals is inhibited in greater degree by the serum of the rabbit than by that of the dog. Since the anti-body of one species has no specific relation to the leucoprotease of the same species, the experiments tend to support the belief that the leucoprotease of different mammalian species is identical.

The enzyme from the rabbit is much weaker than the similarly prepared enzyme from the dog; the anti-enzyme of the rabbit's serum is, on the contrary, much more active. Since experiments previously described have shown that suppuration with solution of fibrin and of necrotic tissue in the dog is associated with loss of anti-enzyme in the purulent exudate, it is not improbable that the well-known absence of typical suppuration with liquefaction of tissue in the rabbit is due to the weakness of the enzyme present in the polynuclear leucocytes and to the strength of the anti-body which opposes it.

Anti-leucoprotease in the Serum of Birds.—An experiment in which the anti-enzymotic power of pigeon's serum was compared with that of dog's, cat's, and goat's serum showed that the serum of the pigeon failed to prevent active proteolysis caused by dog's leucoprotease. The inhibiting action of hen's serum was further

tested with leucoprotease of dog and was compared with that of dog's serum. The following experiment shows the effect of 0.5 and of 1 cubic centimeter of these sera on twenty milligrams of dog's leucoprotease when allowed to digest coagulated serum under the conditions already described:

	Dog's serum.	Hen's serum.
With 0.5 c.c. serum	9.5 c.c.	14.65 c.c.
With 1 c.c. serum	6.05 c.c.	13.75 c.c.

Twenty milligrams of enzyme in the absence of serum caused digestion of coagulated proteid represented by 16.8 c.c. of 1/10 N. sulphuric acid, the control being 2.1 c.c.

It is evident that the anti-enzymotic action of the serum of both pigeon and of hen for leucoprotease of dog, though appreciable, is slight. Since sera of these birds differ from the mammalian sera examined, it has suggested itself that the enzymes present in the leucocytes of the bird might have peculiarities corresponding to this difference. To obtain inflammatory exudates containing leucocytes, aleuronat was injected into the peritoneal cavity of the hen, but from the resulting exudate a sufficient quantity of cells could not be obtained. Turpentine injected into the subcutaneous tissue caused necrosis and accumulation of a considerable number of leucocytes, but suppuration with softening of the tissue did not occur. A powder prepared by the method previously mentioned from the white fibrinous exudate at the seat of inoculation caused very weak proteolysis, when allowed to act at body temperature upon coagulated proteid in the presence of acid, but failed to digest in the presence of an alkaline reaction. Since previous experiments[23] have shown that the bone marrow of the dog contains that enzyme, namely, leucoprotease, which is characteristic of the polynuclear leucocytes, the proteolytic action of a suspension of cells of the bone marrow from the hen was tested in the presence both of acid and of alkali. The bone marrow removed from the bones was shaken violently in salt solution and forced through a fine sieve; the cells thus obtained were washed several times by centrifugalization and suspended in nine times their volume of salt solution. Five cubic centimeters of this suspension were allowed to act on coagulated proteid at body temperature in

[23] *Loc. cit.*

the presence of 0.2 per cent. acetic acid and of 0.2 per cent. sodium carbonate, and with the reaction of the medium unchanged. The experiments of Hedin and of one of us have shown that while the spleen of various mammals undergoes more active autolysis and digests foreign proteid more energetically in an acid medium, it contains at the same time no inconsiderable quantity of an enzyme which digests in the presence of alkali. The proteolytic action of a suspension of splenic cells from the hen was compared with the similarly prepared suspension of bone marrow from the same bird; for further comparison, a suspension prepared from the liver was used. In the following table the digestion of coagulated proteid produced by these suspensions is represented by centimeters of 1/10 N. acid.

	Bone marrow.	Spleen.	Liver.
With reaction unchanged	3.0 c.c.	3.45 c.c.	2.55 c.c.
With 0.2 per cent. acetic acid	10.7 c.c.	12.15 c.c.	8.6 c.c.
With 0.2 per cent. sodium carbonate	2.85 c.c.	3.05 c.c.	2.35 c.c.
Control	2.15 c.c.		1.8 c.c.

These figures show that the bone marrow of the hen, unlike that of the dog, fails almost completely to cause digestion of proteid in the presence of an approximately neutral or alkaline reaction, but causes active proteolysis in an acid medium. An enzyme similar to leucoprotease of the dog, if present, occurs only in very small quantity. The spleen likewise causes very trivial digestion in an alkaline or neutral medium, but is active in an acid medium; the control for the spleen which is lacking doubtless closely approximates that of the bone marrow. The liver which in an acid medium causes less digestion than bone marrow or spleen almost completely fails to digest in a neutral or alkaline medium. An enzyme which digests in an alkaline medium, and occurs in abundance in the organs of mammals is according to the foregoing observations almost wholly wanting in the tissues, notably in the blood-forming organs of the bird.

The absence in the blood-forming organs of the hen of this enzyme is apparently due to its absence in the polynuclear leucocytes. By injecting turpentine into the peritoneal cavity of the hen, a sterile inflammation results: in two experiments at the end of three

and of four days the cavity was found to contain yellowish fluid, but the intestines are loosely matted together by a white or pale greenish, gelatinous material in which are numerous opaque yellow spots. This exudate consists of fibrin holding serum in its meshes; the characteristic polynuclear leucocytes of the bird are present in large number. Bits of this fibrinous exudate washed with salt solution as free as possible of serum were suspended in acid, neutral, and alkaline solutions of which the volume was brought to five cubic centimeters by addition of .85 per cent. salt solution. After digestion at 37° C. during seven days the result of the experiment was as follows:

	Condition of fibrin.
With 0.2 per cent. acetic acid.	Fine powdery sediment.
With 0.1 per cent. acetic acid.	Much eroded.
In normal salt solution.	Unchanged.
With 0.1 per cent. sodium carbonate.	Unchanged.
With 0.2 per cent. sodium carbonate.	Unchanged.

While the experiment does not demonstrate with certainty that solution of fibrin in the presence of acid is due to enzymotic action, the result agrees with that obtained with bone marrow and demonstrates that autolysis of fibrinous exudate containing polynuclear leucocytes fails to occur in a neutral or alkaline medium. The fibrinous exudate obtained several days after injecting turpentine into the pleural cavity of the dog when washed free from serum undergoes complete autolysis, if immersed in 0.2 per cent. sodium carbonate and kept at body temperature.

CONCLUSIONS.

The inhibiting action of the blood serum upon the enzyme of the polynuclear leucocytes, leucoprotease, is exerted by the albumin fraction of the serum. The albumin fraction contains no proteolytic enzymes.

The globulin fraction of the serum contains no anti-enzyme for leucoprotease; it contains, on the contrary, an enzyme which digests proteids in a neutral or alkaline medium. This enzyme resembles leucoprotease which is present in the polynuclear leucocytes of an inflammatory exudate and in the bone marrow from which these cells are derived, and is doubtless identical with the similar enzyme

occurring in smaller quantity in the spleen. This enzyme which is present in the blood serum is held in check by its anti-enzyme, but the latter is in such excess that the serum as a whole is capable of checking the action of leucoprotease when added in considerable quantity.

Leucoprotease of one mammalian species is inhibited by sera of other mammalian species, but the anti-enzymotic activity of various sera differs; the anti-enzyme of the rabbit's serum is stronger than that of dog's serum, when tested either with dog's or with rabbit's leucoprotease. The co-existence in the rabbit of leucoprotease with feeble strength and anti-body of great activity may explain the absence in these animals of typical suppuration with liquefaction of tissues.

The serum of birds which have been tested, namely, pigeon and hen, almost completely fails to inhibit mammalian leucoprotease (of dog). The polynuclear leucocytes, the bone marrow and the spleen of the hen do not contain an enzyme resembling leucoprotease of mammals. The absence of anti-enzyme in the serum is associated with absence of a corresponding enzyme in the leucocytes.

ON THE ELECTRICAL CHARGE OF THE NATIVE PROTEINS AND THE AGGLUTININS.

By CYRUS W. FIELD[1] and OSCAR TEAGUE.

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In a previous paper[2] it was shown that the particles of both toxin and antitoxin wandered under the influence of an electric current toward the cathode and that the reaction (acidity or alkalinity) of the solvent did not influence the direction of migration. Since Hardy[3] and Pauli[4] demonstrated that the proteins which they used were amphoteric, *i. e.,* that they pass toward the anode in an alkaline medium and to the cathode in an acid one, there has been a tendency to generalize by assuming that all proteins behave in this manner. If such were the case, we pointed out, it would follow from our experiments that toxin and antitoxin are not true proteins. At the same time, however, we mentioned that from the few experiments in which this question had been considered, the protein matter of the broth or serum seemed in every instance to travel with the toxin or antitoxin toward the cathode. Further experiments have confirmed this result. It was also shown that the protein of normal horse serum and of non-toxic broth travels toward the cathode. Hence our work offers as yet no evidence either for or against the view that toxin and antitoxin are non-protein in nature.

We maintain that the results which Hardy and Pauli obtained, working with denaturalized proteins, are in no wise applicable to the native proteins, but that these carry a distinct electrical charge and are not amphoteric. We are here in accord with Iscovesco[5] and his co-workers, who investigated the charge of colloids con-

[1] Assisted by a grant from the Rockefeller Institute for Medical Research.

[2] Field and Teague, *Journal of Exper. Med.,* 1907, viii, 86.

[3] *Jour. of Physiol.,* 1899, xxiv, 288.

[4] *Hofmeister's Beit.,* 1906, vii, 531.

[5] *Compt. rend. Soc. biol.,* 1906, lxi, 195, 355, 378, 470, 568.

tained in various body fluids. Their method consisted in treating the fluid with electro-negative (arsenic sulphide) and electro-positive (ferric hydrate) inorganic colloids and their conclusions were based upon the fact that colloids of opposite sign when brought together form precipitates. Thus they found that the peritoneal fluid of the horse contains only electro-positive colloids, while the pericardial fluid contains those of both signs; that blood plasma contains both positive and negative albumins with positive and negative globulins, whereas the serum contains only the positive globulin along with albumins of both signs; that the fluid of a tubercular abscess deprived of its leucocytes contains only electro-negative colloids; that the amniotic fluid contains both positive and negative albumins, but only negative globulins. From these experiments Iscovesco concludes that there are no colloids which do not bear a distinct electrostatic charge.

Since our method gave no indication of the presence of an electro-negative albumin in normal serum, we are inclined to believe that Iscovesco by his manipulations produced a change in sign of the charge carried by certain proteins and that all of his findings are therefore not applicable to the proteins originally present in the fluids he investigated.

In our previous work with tetanus toxin we investigated only the tetanospasmin and its antibody; we have since shown by testing the agar extracts for their lytic or antilytic action on horse cells that both tetanolysin and antitetanolysin travel toward the cathode under the influence of an electric current. Having determined the electrical charge of toxin and antitoxin, we next applied the same method to an investigation of the agglutinins.

The agar was divided into one centimeter lengths; the agglutinin was found to have traveled seven centimeters into the cathode agar, the anode agar remaining free of agglutinin. The first centimeter length was extracted with five cubic centimeters of water and this extract would still agglutinate at a dilution of 1:100.

The specific agglutinins investigated travel toward the cathode. These results are diametrically opposed to those of Biltz, Much and Siebert,[6] who are the only workers, so far as we know, who

[6] *Zeit. für diatet. und phyckal. Ther.*, 1905, viii, 19.

have investigated this subject. They passed a current through lacto-serum contained in a U-shaped tube for from one half to one hour, and found that the fluid around the anode agglutinated at 1:20, that around the cathode not at all, and that from the middle of the U-shaped tube at 1:8. Normally the serum agglutinated at 1:4. They state that after the passage of the current the fluid from around the anode was 1/10 normal acid. We would expect this amount of acid to agglutinate at approximately 1:20, since 1:200 represents about the flocking limit of hydrochloric acid for bacteria.

As stated in a previous article, we took special precautions to eliminate the products of electrolysis. However, to show conclusively that it was the specific agglutinin, and that alone, which was responsible for the agglutination in our experiments, the extracts were also tested against other bacilli than those which were agglutinated by the serum under investigation.

TABLE I.

STRENGTH OF ELECTRIC CURRENT 110 VOLTS; ½ TO 1 MILLI-AMPERE.

Serum agglutinating the typhoid bacillus at 1:2000. Current passed for six hours.

Organism.	Cathode Agar cm. Lengths.											Anode Agar.	
	1	2	3	4	5	6	7	8	9	10	11–20	1–10	11–20
B. typhosus.	+++	+++	+++	+++	++	++	+	o	o	o	o	o	o
B. coli.	o	o	o	o	o	o	o	o	o	o	o	o	o
Shiga's bacillus.	o	o	o	o	o	o	o	o	o	o	o	o	o
Para typhoid b.	o	o	o	o	o	o	o	o	o	o	o	o	o
Biuret react.	+	+	+	+	+	+	trace						

NOTE.—As one centimeter lengths of the anode agar showed no agglutinin in repeated experiments we have here tested extracts from ten centimeter lengths.

If the agglutination were due to the presence of products of electrolysis we would expect the other bacilli to be agglutinated as well as typhoid. Such, however, was not the case. Hence, we believe that we have shown conclusively that the agglutinins travel toward the cathode.

It has been shown by Bechhold[7] and Buxton, Schaeffer, and Teague[8] and others that bacteria move toward the anode under

[7] *Zeit. physik. Chem.*, 1904, xlviii, 385.
[8] *Ibid.*, 1906, lvii, 47.

the influence of an electric current, that is, they carry a negative charge.[9] Our findings with regard to the agglutinins is therefore especially interesting, since it shows that in the phenomenon of agglutination we have the combination of an electro-negative suspension with an electro-positive colloidal solution. Since ions of opposite sign are essential for a chemical reaction and colloids of opposite sign when brought together form precipitates, our results harmonize with both the chemical and the colloidal view of the phenomenon.

Bacteria which have been saturated with agglutinin and then washed in a number of changes of water until the wash water contains no more agglutinin were placed in the cell and after eight hours the agar was tested for agglutinin. A small amount was found in the cathode agar showing that under the influence of the electric current the agglutinin bacteria combination was disassociated and that the agglutinins passed to the cathode. Bacteria have been disassociated from agglutinins by other means,[10] but so far as we are aware, this is the first time that disassociation has been affected by means of the electric current.

CONCLUSIONS.

1. Tetanolysin and antitetanolysin travel toward the cathode under the influence of an electric current.

2. The specific agglutinins are electro-positive.

3. The proteid matter of serum is not amphoteric but travels toward the cathode whether its reaction be acid, neutral, or alkaline.

4. The bacteria-agglutinin combination may be disassociated by means of the electric current.

[9] Cernovodeanu and Henri (*Compt. rend. Soc. de Biol.*, 1906, lxi, 200) claim that dysentery bacilli travel toward the cathode but we have not found this to be the case.

[10] Quoted by Eisenberg in *Cent. f. Bakt*, 1906, xxxi, 540 are the following: Joos (if fresh bacilli are added to agglutinated bacilli, which have been previously washed free from serum, the former are agglutinated), Landsteiner and Jagio and Landsteiner and Reich (dissociation at high temperatures).

HETEROTRANSPLANTATION OF BLOOD VESSELS PRESERVED IN COLD STORAGE.

By ALEXIS CARREL.

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For the purposes of the transplantation of blood vessels in man and animal, a method which will suffice to prevent for a time the disintegration which extirpated tissues quickly undergo, would be of great value. With the view of retarding the occurrence of these cadaveric changes, I kept in cold storage for several days blood vessels resected from dogs; and afterwards I transplanted these vessels to cats. It is well known that the tissues of an animal do not grow, or grow hardly at all in an animal of another species. Nevertheless, these heterotransplantations were attempted with the aim of ascertaining whether the vessels, in spite of the toxic action of the cat's blood on the dog's tissue, could adapt themselves to the new conditions of life, and take over the function of the vessels removed. Five experiments were performed.

Experiment I. Female Cat.—A segment of the abdominal aorta located between the mouths of the renal and ovarian arteries was resected. The circulation in the lower part of the aorta was re-established by interposing a segment of the external jugular vein between, and suturing it to the cut ends of the aorta. This segment of the jugular vein had been extirpated from a dog seven days before and kept in an isotonic solution of sodium chloride at or about the freezing point. But the temperature of the refrigerator was not constant, and sometimes varied from 32° F. to 40° F. After the operation was completed, the pulsations of the femoral arteries and of the arteries of the hind limbs were normal. On the second day, the hind limbs became hyperæsthetic. On the third day, paralysis of the hind feet appeared, and a few hours later paralysis of the hind limbs was noted. At the same time, the pulsations of the femoral arteries completely disappeared. On the following days, the paralysis diminished progressively. Twenty-two days after the operation, the animal could walk almost normally. But the pulsations of the femoral arteries did not reappear. By direct examination it was found that the transplanted venous segment was surrounded by a thick layer of vascular connective tissue and its lumen was completely obliterated.

Experiment II. Female Cat.—A segment of the abdominal aorta of a large cat was resected and replaced by a segment of carotid artery which had been extirpated from a young dog twenty days before, and kept in an isotonic sodium chloride solution at about 32° F. Sometimes the temperature of the refrigerator rose for a few hours to 40° F., or even 44° F. After the operation, the pulsations of the femoral arteries and the movements of the hind limbs remained constantly normal. Forty-eight days after the operation, the animal was etherized and its abdomen opened. The pulsations were found normal in the abdominal aorta, and in the segment of carotid. The location of the anastomosis was marked by a slight hardening of the arterial wall. There was little reaction of the connective tissue around the transplanted vessel. The wall of the carotid appeared to be less elastic than that of the aorta. No dilatation of the transplanted segment was observed. The abdomen was closed after the examination, and the animal kept alive. Now, seventy-seven days after the transplantation, the pulsations of the femoral arteries are normal and the animal is in excellent condition.

Experiment III. Male Cat.—A large cat was subjected to an operation similar to the last one. The segment of carotid interposed between the cut ends of the aorta had been extirpated from a dog three days before and kept in dog's defibrinated blood at 32° to 34° F. After the operation, the pulsations of the femoral arteries and the movements of the hind limbs remained constantly normal for thirty-four days. On the thirty-ninth day after the operation it was found that the pulsations of the femoral arteries had disappeared and that both anastomoses were occluded by a small deposit of fibrin. In the lumen of the transplanted segment was a soft clot of recent formation. The thrombosis appears to be due merely to an inclusion and proliferation of the external sheath of the carotid in the line of suture.

Experiment IV. Male Cat.—A small piece of the abdominal aorta of a large cat was resected, and a short segment of carotid artery was interposed between the cut ends. The arterial segment had been extirpated from a dog seventeen days before and kept in defibrinated dog's blood at 32° to 34° F. Ten days after the operation, the pulsations of the femoral arteries became weaker and disappeared completely. By direct examination it was found that the upper anastomosis was obliterated by a small deposit of fibrin. The upper part of the transplanted segment was filled with a clot of recent formation. The lower anastomosis was excellent. The endothelium and the wall of the segment of the carotid were apparently normal.

Experiment V. Male Cat.—An operation similar to the last one was performed on a large cat. The segment of carotid interposed between the cut ends of the aorta had been extirpated from a dog four days before and kept in Locke's solution at 32° to 34° F. After the operation, the animal remained in normal conditions. He was killed six days after the operation. The union of the transplanted segment to the aorta was apparently perfect. There was no deposit of fibrin at the lines of suture and the wall of the segment of carotid artery was apparently normal.

CONCLUSIONS.

The experiments show merely that blood vessels transplanted from dog to cat can act as arteries for seventy-seven days at least; and that having spent several days in cold storage does not interfere with their ordinary functions. The animals operated upon must, however, be kept under observation for several months, or, indeed, for several years, before any conclusion can be drawn concerning the practicability of this method of preservation and heterotransplantation of blood vessels.

NUCLEIN METABOLISM IN A DOG WITH ECK'S FISTULA.

By J. E. SWEET and P. A. LEVENE.

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All nitrogenous substances completely metabolized in the organism of mammals are removed by the urine in form of urea. Every other nitrogenous constituent of the urine is either a substance which has for one reason or another escaped its final transformation, or has not been metabolized by the organism at all. Under metabolism here, of course, is understood a set of chemical reactions which result, first, in maintenance of the integrity of the body's tissue and, second, in supplying it with calories sufficient to perform all the functions of the organism.

The quantity of nitrogen metabolized by the organism for the purpose of energy production is regulated primarily by the intake of nitrogenous food-stuffs, and by the quantity of reserve protein deposited in the organism. The quantity of nitrogen metabolized as a result of tissue deterioration for the purpose of maintaining its integrity is determined by the mass of the cells in the organism, by the amount of work which the tissues are called upon to perform, and by the intensity of the effort employed by the organism in performing the work. Thus, work of short duration, but of such intensity as would occasion a state of fatigue, brings about greater tissue deterioration than prolonged work of lower intensity.

Under normal conditions there exists a definite proportion between the two phases of nitrogenous metabolism, namely, between metabolism of nitrogenous food-stuffs of reserve protein on the one hand, and metabolism of cellular protein on the other. This proportion changes with the change of the plane of nutrition of the organism and with change of the quantity of protein in the food. However, conditions are conceivable in which it is made possible that the organism may be supplied with a sufficient amount of

energy by the sacrifice of the tissue integrity, or conditions may exist in which the tissue integrity is maintained by an unusually high consumption of reserve protein. Thus it is important to be able to trace separately the two phases of nitrogenous metabolism.

Analysis of the intake of food stuffs, a record of heat production, estimation of carbon dioxide and of urea removed by the organism furnish the data required to follow energy production. But one is confronted with considerable difficulty when he attempts to obtain information regarding the condition of the cellular elements of the tissues. This is due to the fact that deteriorated tissue elements are utilized for the purpose of metabolism in the same manner as the protein of the food or as the reserve protein. Their nitrogen is removed in the form of urea. Some components of the cellular elements, however, have a chemical composition distinctly different from that of the reserve protein. These substances, previous to their transformation into urea, undergo a cycle of chemical reactions which is different from that of the reserve protein; the intermediate products of their metabolism differ from those of the latter, and it is very probable that the mechanisms regulating the transformation of the two groups of substances are independent of one another. Indeed, in the light of this assumption one can readily explain the fact that transformation of special components of tissue elements varies considerably in different species, although transformation of simple reserve-protein occurs in an identical manner, and results in the same production of energy.

Among such components of tissue elements special interest is attached to the nucleins, since they constitute the principal substance of the nuclei of all cells. The attempt has been repeatedly made to ascertain the degree of tissue deterioration by analysis of the nuclear derivatives in the urine, the substances of special interest being uric and phosphoric acids. It has been established in recent years that uric acid is formed in the organism through the oxidation of purin bases, which are the principal components of nucleic acids. But uric acid is metabolized further in the organism of mammals, that is, it is oxidized and is finally removed from the organism in the form of urea. Uric acid detected in the urine represents only a fraction of the acid formed in the organism, namely, that part

which has escaped further oxidation. Hence satisfactory information regarding cellular disintegration cannot be gained by the investigation of uric acid output under normal conditions; but the results of uric acid analysis can be made valuable for the solution of the problem, if conditions are found under which the further transformation of uric acid can be prevented.

Clinical observations, and the studies of Pawlow, Nencki, Hahn and Massen especially, have suggested the probability that the liver is the principal organ concerned in the oxidation of uric acid. On the other hand, recent work of Weiner, Burian, Jones and Schittenhelmn have shown that many other animal tissues possess the same power. It must be borne in mind that the Russian investigators were not engaged in the study of purin metabolism especially, and their observations were made at a time when uric acid formation was not yet clearly understood. The facts make a revision of the older work on purin metabolism very desirable.

The present investigation was carried out on a dog in which an anastomosis between the portal vein and the inferior vena cava had been successfully performed by one of us (Sweet). The purposes of investigation were: (1) To ascertain whether or not the output of uric acid was above normal, if the dog was maintaining nitrogenous equilibrium, and was keeping its original weight. (2) To ascertain whether or not the output of uric acid was increased markedly after administration of nuclein or of its derivatives (nucleoproteid of the mammary gland of the cow, nucleic acid of fish sperm, adenin sulphate, and thymin were employed). (3) To ascertain whether the animal possessed the power to metabolize thymin. (4) To ascertain whether or not the output of uric acid was increased on a diet poor in protein material, although containing carbohydrates and fat in quantities sufficient to supply the organism with the required amount of calories. (5) To ascertain whether or not the output of uric acid was increased during starvation.

The present communication represents the results of experiments performed on one dog, and cannot, therefore, serve as a basis for broad generalization. The work is communicated in its present incomplete state, because accidental circumstances render its im-

mediate completion unpracticable; the results already obtained are believed to be of interest.

Methods.—The urine was collected in three-day periods, the dog being catheterized at the beginning and at the end of each period. The receptacle contained a quantity of 10 per cent. solution of sulphuric acid to make up with the urine a solution of about 2 per cent. of sulphuric acid. No other preservative was added.

The nitrogen estimations were made by the Kjeldahl-Gunning method. The phosphorus was estimated gravimetrically, the urine being evaporated and fused with a mixture of potassium carbonate and sodium nitrate. Uric acid was estimated by the Leube-Salkowski method, ammoniacal silver chloride being used instead of the nitrate.

Elimination of Uric Acid.—Information regarding uric acid elimination by dogs with Eck's fistula is limited to the observations of Pawlow, Nencki, Hahn and Massen. Their dogs were fed on a mixed diet, no special attention being paid to the quantity of purin bodies in the food-stuffs, and no special care being exercised in collecting the daily quantities of urine. Moreover, since the dogs used by the Russian investigators developed the typical nervous attacks described by Pawlow, without further evidence the objection might be urged that the high uric acid output was occasioned by deterioration of the nuclear elements of the liver, or was secondary to the convulsive attacks.

In the present work the experiment was not begun until the nutrition of the dog after operation was so much improved that it maintained nitrogenous equilibrium. Furthermore, the dog was fed on cracker dust, plasmon, lard, and salt, to eliminate all purin in the diet, and had been on this diet for several weeks prior to the beginning of our records. It is, therefore, obvious that all the uric acid found in the urine must have originated in disintegration of tissue. The output of uric acid in this experiment was considerably higher than that of a normal dog.

Feeding of Nucleoprotein.—On two successive days the dog received, instead of plasmon, twenty-five grams of nucleoprotein of the mammary gland of the cow. As seen from the table, a slight increase in the uric acid output occurred, but as the nucleoprotein

FEMALE MONGREL SPANIEL—OPERATION, MARCH 5, 1906.

Date.	Dog Received.	Weight.	Total N-Intake.	Total N-Output.	N in Urine.	N in Faeces.	Total Uric Acid.	Ingested P_2O_5	P_2O_5 in Faeces.	P_2O_5 in Urine.	Amount of Urine.	Weight of Dry Faeces.
Apr. 27–28–29.	75.0 g. Plasmon, 300.0 g. Cracker dust, ca. 35.0 g. Lard.	9250	12.891	14.140 g.	12.909	1.231 g.	0.240 g.	3.9045 g.	—	2.826 g.	1500	38.0 g.
30–May 1–2.	75.0 g. Plasmon, 300.0 g. Cracker dust, ca. 35.0 g. Lard.	9400	"	14.867	12.18	2.687	0.492	"	1.3278 g.	2.254	2020	88.0
3–4–5.	25 g. Plasmon, 300 g. Cracker dust + 50 g. Nucleoproteid.	9250		14.226	12.78	1.446	0.663		—	2.157	1700	45.0
6–7–8.	Plasmon, Cracker dust, Lard.	9250	"	12.559	11.2	1.359	0.230	"	—	2.113	1000	43.0
*16–17–18.	" + 1 g. Adenin sulphate.	9250		12.118	10.69	2.028	0.620		—	2.317	2190	65.0
19–20–21.	Plasmon, Cracker dust, Lard.	9150	"	14.631	13.8	0.831	1.050	"	—	3.156	3000	43.0
22–23–24.	" + 10 g. Nucleic acid.	—		15.13	13.37	1.766	1.301		2.519	3.947	3420	68.0
25–26–27.	Plasmon, Cracker dust, Lard.	9200	"	13.75	12.53	1.22	0.681	"	—	2.632	2710	58.0
28–29–30.	" " "	—	"	13.804	12.64	1.164	0.721	"	—	2.644	2560	38.0
31–June 1–2.	" + 3 g. Thymin.	9200		12.512	11.22	1.292	0.707		—	2.408	3005	45.0
3–4–5.	Plasmon, Cracker dust, Lard.	9300	"	14.106	11.986	2.12	0.644	"	—	2.596	3020	58.0
6–7–8.	" + 6 g. Thymin.	9350		12.874	11.708	1.166	0.755		—	2.940	4425	43.0
9–10–11.	Plasmon, Cracker dust, Lard.	9400	"	12.602	11.401	1.201	0.796	"	—	2.517	2800	38.0
12–13–14.	" " "	9550	"	13.542	11.835	1.617	0.840	"	—	2.690	2720	58.0
15–16–17.	" " "	9650	"	14.068	12.513	1.555	0.797	"	—	2.382	2800	60.0
18–19–20.	" " "	9600	"	14.079	12.699	1.380	0.773	"	0.9322	2.467	2650	48.0
21–22–23.	195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	9700	3.042	9.467	8.808	0.659	0.947	0.774	—	1.846	2800	28.0
24–25–26.	195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	—	"	7.638	7.140	0.498	0.972	"	—	1.622	3260	23.0
27–28–29.	195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	10350	"	12.428	8.708	3.720	1.025	"	—	1.902	3590	285.0 †
30–July 1–2.	195 g. Cane sugar, 225 g. Cracker dust, Lard as above.	11200	"	10.824	7.382	3.442	0.857	"	—	1.294	2700	422.0 †
3–4–5.	Starvation.	8700	0.0	7.344	7.344	No faeces.	0.906	None.	0.0	1.518	3200	0.0
6–7–8.	"	8350	0.0	6.65	6.65	No faeces.	0.746	None.	0.0	1.390	1450	0.0

75.0 g. Plasmon (3 days) = 8.835 g. N.; = 2.8725 g. P_2O_5.
300.0 g. Cracker dust (3 days) = 4.056 g. N.; = 1.032 g. P_2O_5.

* May 9–15 not worked out, owing to accidental loss of urine.
† Faeces does not dry thoroughly, owing to large amount of sugar contained.

employed in the experiment had been extracted with alcohol and dried, its absorption and assimilation were perhaps somewhat unsatisfactory. Few experiments in which dogs have been fed on pure nucleoproteins are recorded, but the feeding of organs rich in nucleoproteins and purins in the experiments which have been reported have failed to cause any marked increase in the elimination of uric acid.

Feeding of Adenin.—One gram of adenin sulphate was administered the first day of the experiment. All earlier experiments in which dogs were fed on purin bases failed to show any increase in uric acid output. Only Minkowski[1] records an increased elimination following xanthin or hypoxanthin feeding, but he failed to obtain an increase with adenin. In the present experiment a slight increase in the uric acid output was noticed during the period of feeding; it was more marked in the following period. It is difficult to determine from this one experiment whether the rise was actually occasioned by the administration of adenin or occurred accidentally. Observations are recorded in which increase of uric acid appeared a few days after the administration of nuclear material (Camerer[2]). The increase in uric acid elimination during the period of adenin feeding, and during the following period was 0.3668 gram, an amount equal to 44.27 per cent. of the administered adenin. There was also noted an apparent retention of nitrogen during the period of the experiment, and an increase in the period next following, while during the latter period an increase in the uric acid elimination and diuresis were noted. All these findings may be explained by the action of adenin on the kidneys.

Feeding with Nucleic Acid.—The dog received in addition to its usual food ten grams of the sodium salt of nucleic acid of fish sperm. There was a marked increase of uric acid, an increase of phosphoric acid elimination and increased diuresis. The increase of nitrogen elimination did not exceed 0.8 gram. The nitrogen of this increase apparently had its origin in the absorbed nucleic acid, and corresponded to about 4.8 grams of nucleic acid. In reality, however, part of the increase in nitrogen elimination was occasioned by the

[1] *Arch. f. exper. Path. u. Pharm.*, 1898, xli, 375.
[2] *Zeitsch. f. Biol.*, 1896, xxxiii, 139; 1897, xxxviii, 206.

higher diuresis. According to the analysis of Levene and Mandel,[3] the nucleic acid employed in this experiment contained not more than 0.4 gram of purin bodies. The increase of uric acid elimination was about 0.2000 gram, which represents about 40 per cent. of the purin ingested with the nucleic acid.

In normal dogs an increase in the output of uric acid after nucleic acid feeding is recorded by Minkowski.

Feeding of Thymin.—Feeding experiments with pyrimidin bases have been performed by Stendel[4] with the purpose of studying the synthetic formation of uric acid in the living organism. Administration of thymin remained without influence on the output of uric acid. Stendel's efforts to recover the substance in the urine were unsuccessful.

In the present experiment, the dog received six grams of thymin, which was added to his food. No increase in the total output of nitrogen was noted; there was rather a slight nitrogen retention. On the other hand, a marked diuresis occurred, and the urine contained about 13.5 grams of the ingested thymin.

In order to obtain thymin, one liter of the urine was rendered acid by means of nitric acid and treated for pyrimidin bases by the Kossel-Jones method. The substance obtained in this manner was recrystallized out of very dilute sulphuric acid. A free crystalline base showing all the properties and the appearance of thymin was obtained. The substance had the following composition:

0.1372 grams gave 27.4 c.c. of nitrogen (over 50 per cent. potassium hydroxide) at $t° = 32.0°$ C. and $p = 756$ mm.

For $C_4H_6N_2O_2$:

Calculated $N = 22.22$ per cent.
Found $N = 22.96$ per cent.

Thus the greater part of the ingested thymin is eliminated by the kidneys; but it is difficult to form an opinion as to the extent of the decomposition in the organism, since it is possible that only part of the thymin had been absorbed, while another part may have suffered decomposition in the intestinal tract, through the action of bacteria.

[3] *Zeitsch. f. physiol. Chem.*, 1906, l, 1.
[4] *Ibid.*, 1901, xxxii, 285.

Since it had been observed that the oxidation of thymin in the organism of the dog employed in this experiment was greatly impaired, an attempt was made to discover thymin in the urine obtained during the purin free periods, and also during the periods following the nucleic acid feeding. This was done with a view to establish the extent of nucleic acid decomposition in the organism. It was expected that thymin would be found in the urine, if considerable quantities of it were formed in the organism. From the experience of one of us (Levene[5]) with autolysis of animal organs, and also from that of Jones[6] and of Reh,[7] it is known that on disintegration of tissues, pyrimidin bases are formed. Nevertheless, neither during the period of nuclein free diet nor during that of the nucleic acid feeding, did the urine show any traces of thymin. One is, therefore, led to the conclusion that in the living organism nucleic acid either does not suffer complete disintegration, or disintegrates slowly, the small quantities of thymin thus formed being further decomposed.

Diet with a Low Protein Content.—The object of these experiments was to obtain further information regarding the factors which regulate the output of endogenous uric acid. Our present knowledge of endogenous uric acid is based on a very limited number of observations made on men. On the normal dog the study of nuclein metabolism has not been possible, since the intermediate products of decomposition of these substances are very readily transformed by the animal into urea. However, a dog with Eck's fistula possesses a lower degree of oxidizing power for purin bases than even man. Thus, it is seen, after feeding adenin that nearly 45 per cent. of the substance was eliminated with the urine in form of uric acid. Burian and Schur[8] found in men, after administration of hypoxanthin, a figure approaching this, and one less than half so high after feeding other purin bodies. It is believed by most observers that the output of endogenous uric acid remains practically constant and is totally independent of the quantity and of the quality of the food taken.

[5] *Zeitsch. f. physiol. Chem.*, 1901, xxxii, 546; 1903, xxxvii, 521; 1904, xli, 402.
[6] *Ibid.*, 1904, xlii, 35.
[7] *Hofmeister's Beiträge*, 1903, iii, 569–573.
[8] *Pflüger's Arch.*, 1900, lxxx, 342.

True, Burian and Schur,[9] the principal advocates of this view, considered it necessary to point out that extreme changes in the quantity or in the character of the food may alter the output of endogenous uric acid. Excessive intake of food, which taxes the gastrointestinal glands beyond normal, may result in an increased uric acid elimination, while on the other hand, starvation results in a decreased uric acid output, since it lowers the intensity of general metabolism. Folin[10] much more emphatically expresses the view that the output of uric acid is not invariably the same for an individual, as Burian and Schur[11] claim.

Folin arrives at this conclusion: "When the total amount of protein metabolism is greatly reduced, the absolute quantity of uric acid is diminished, but not nearly in proportion to the diminution in the total nitrogen, and the percentage of uric acid nitrogen in terms of the total nitrogen is, therefore, much increased." L. B. Mendel[12] states, "When the total amount of protein metabolism is greatly reduced, the endogenous output of uric acid is diminished, though this is not the case within ordinary ranges of diet."

A reduction of the intake of albuminous food-stuff should occasion for a time an increased disintegration of the body proteins, and with it a greater destruction of the cellular elements of the tissues. This, in its turn, should result in a greater formation of uric acid. Therefore, it seemed to us probable that the diminished uric acid output, under the conditions indicated by Folin, Mendel, and others, was caused, not by diminished formation, but by more complete combustion of the purin bodies. *A priori*, it might be expected that the organism utilized more completely nitrogenous substances of non-protein nature, when there was a lack of protein in the food. If this assumption were correct, one should find under these conditions an increased elimination of uric acid when the power of the organism to consume purin bodies is diminished. In our experiments, indeed, the diminution of the protein intake and its substitution by fat and carbohydrate (so that the intake of

[9] *Loc. cit.*
[10] *Amer. Jour. of Physiol.*, 1905, xiii, 87.
[11] *Loc. cit.*
[12] Harvey Lecture, *Journal of the American Med. Ass.*, 1906, XLVI, 843, 944.

calories was not altered) was followed by an increase in the uric acid elimination. It was expected that the organism would, on a continued low protein diet, finally adjust itself to the condition and preserve the integrity of the remaining cellular elements. In our dog, the uric acid elimination suffered a marked fall during the fourth low protein period.

Starvation.—In order to understand fully the influence of diet on the output of endogenous uric acid it was considered necessary to ascertain the influence of starvation on uric acid elimination. It was originally planned to have the starvation period precede the period of low protein diet. Since fasting might prove fatal to the dog, it was thought safer to change the order of the experiments. During the first starvation period, the uric acid elimination showed a rise, as compared with the preceding period. The output, however, was not as high as during the early period of low protein diet. Comparing the starvation periods with the periods of diminished protein diet, one gains the impression that the uric acid output was higher during the latter experiment. One must, however, bear in mind that this experiment preceded starvation and the continued low protein diet caused a considerable diminution in the mass of the cellular elements of the body. On the other hand, it is also possible that the absence of activity of the digestive glands is the cause of the lower output of uric acid during starvation.

The Period Following Starvation.—After the second starvation period the animal received the usual diet, consisting of cracker dust, plasmon, and lard. It ate all the food given, but on the next day refused to eat, and later developed the typical symptoms described by Pawlow. Once, typical epileptic convulsions were observed by the writers. The animal remained for forty-eight hours without food, and showed a tendency to recover. It refused the usual food—plasmon, etc.—and also milk, and was then given boiled meat which it ate ravenously, apparently improving. However, after eighteen hours, it again relapsed into the nervous condition described, and was found dead on the morning of the fifth day after the end of the starvation period.

The autopsy showed a condition of extreme emaciation. Aside from cachexia, the organs of the body presented a normal appear-

ance. The liver was possibly of smaller size than usual, but showed no cirrhotic or other lesions. The veins of the abdominal viscera were filled with an injection mass, the vena cava having been tied above the diaphragm, and the site of the anastomosis was minutely dissected; no collateral branches, which sometimes carry portal blood around the ligature about the portal vein, were found. The opening of the fistula was large and perfectly formed.

On the Diuretic Action of Nuclein Derivatives.—A review of the tables clearly shows that a more or less pronounced diuresis follows the administration of nuclear material. The tables of Burian and Schur,[13] and those of Minkowski indicate the occurrence of this phenomenon. In the experiments of these investigators, increased diuresis followed only the administration of nucleins, or nuclein containing tissues, but did not follow the administration of the free purin bases. In our experiments diuresis was less pronounced after the administration of adenin. It is worthy of note, however, that the administration of thymin caused the most pronounced diuresis. As is well known, the methylated dioxypurins possess a much higher diuretic action than other purin derivatives, and it seems probable that the methylated dioxypyrimidin also possesses a high diuretic action. This question, however, can be answered only after further study.

[13] *Loc. cit.*